

Materials and Methods Relating to Treatment of Injury and Disease  
to the Central Nervous System

FIELD OF THE INVENTION

- 5 The present invention relates to novel methods and materials for treating injury and disease of the central nervous system (CNS). Particularly, but not exclusively, the invention provides methods and materials for spinal cord regeneration and remyelination following spinal cord injury (SCI), stroke, or disease such as  
10 multiple sclerosis (MS) and epilepsy.

BACKGROUND TO THE INVENTION

- Over 250,000 people in the United States, and several million worldwide, are permanently disabled due to a past spinal cord  
15 injury (chronic SCI), and about 12,000 people are newly injured (acute SCI) in the United States each year. Additionally, paralysis due to SCI is predominantly a condition of the young: 60% of spinal cord injuries occur before age 30, and the most frequent incidence is at age 19. Most injuries are caused by  
20 motor vehicle, sports or work-related accidents, or by violence. Estimated costs of care for SCI patients in the United States alone exceed \$9 billion per year and over \$1.5 million per patient lifetime.
- 25 Following trauma to the adult central nervous system (CNS) of mammals, injured neurons do not regenerate their transected axons. Currently, the mainstay of treatment in spinal cord injury is still rehabilitation. There is little one can do to address the primary injury. After spinal injury, three major  
30 classes of damage have been identified:

1. Neuronal cell death. The death of nerve cells due to injury presents a difficult problem because nerve cells lose the ability to undergo cell division as they mature into the  
35 highly specialized cells that make up our nervous systems.

Some cells die during the traumatic insult, others die in the hours, days or even weeks following injury. Regardless of when the cell death occurs, functional connections cannot be established if the nerve cells no longer exist. 5 Death of glia, also interferes with nerve function. The first therapeutic goal is to preserve as many cells as possible, also known as neuroprotection. Even with neuroprotective drugs or therapies, some nerve cell death is still likely in SCI. Therefore, replacement of nerve 10 cells may be required.

2. **Disruption of nerve pathways.** The long axons in the ascending and descending tracts of the spinal cord undergo Wallerian degeneration after injury. Axonal regeneration 15 must occur to re-establish neuronal circuits.

3. **Demyelination.** Myelin sheaths insulate the long, thin axons to facilitate nerve impulse transmission. In some types of SCI, as well as stroke and epilepsy, the nerve 20 cells and axons may not be lost or interrupted; neuronal dysfunction may be due to loss of the myelin sheath. This type of damage may be the most amendable to treatment because rewiring of complex circuits may not be necessary and remyelination of axons is known to be possible.

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#### **Possible therapies**

##### **1. Replacement of Nerve Cells**

Mature nerve cells cannot divide to heal a wound. Replacement of 30 lost nerve cells would require transplantation into the site of injury with the hope that grafted nerve cells would mature and integrate into the host nervous system. Use of human fetal tissue has shown promise in some studies, however, it presents ethical and technological considerations regarding donor tissues 35 and important questions about immune rejection of transplanted

cells. Very recently, scientists have discovered the presence of adult neural stem cells that can be stimulated to divide and develop into neurons and glia. This exciting finding has opened up new possibilities for cell therapy.

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## **2. Regeneration of damaged axons**

Neurons in both the central (CNS) and peripheral (PNS) nervous systems are intimately associated with glia. After injury, CNS glia largely inhibit regeneration, whilst in the PNS, the Schwann cells facilitate regeneration. The cells are seeded in specially designed "guidance channels" that have been shown to promote the regeneration of nerve fibres in severed rat spinal cords. Schwann cells and neurons produce growth factors. By introducing these factors into injury sites, alone or in combination with grafts, these have shown that they can stimulate additional spinal cord regeneration. Schwann cells can be genetically engineered to produce growth factors, and these also improve regeneration. Some improvement in hind limb motor function have been observed after grafting, however, the results are not reliable enough yet to justify clinical trials of these procedures. Two exciting new studies show that olfactory ensheathing glia can "usher" long nerve fiber growth into surviving spinal cord regions beyond the site of SCI, after these fibers exit a Schwann cell bridge or grow past the site of injury. These promising studies give hope that successful restoration of function after SCI, stroke or epilepsy may occur one day.

## **3. Remyelination of axons**

Schwann cells are the cells in peripheral nerves that form myelin sheaths. They are not usually found in the brain or spinal cord where oligodendrocytes are responsible for myelin production. Researchers have shown that Schwann cells grafted into the brain can myelinate central axons. When the loss of myelin is an important part of an injury, implanting Schwann cells could

stimulate remyelination and perhaps restore function. A multi-center clinical trial has been initiated at other research centers to study a drug (4-AP) that appears to temporally restore signal transmission through demyelinated nerve fibers.

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**CNS myelin and its major inhibitory effects during axonal regeneration**

An important barrier to regeneration is the axon growth inhibitory activity that is in CNS myelin and that is also  
10 associated with the plasma membrane of oligodendrocytes, the cells that synthesize myelin in the CNS. The growth inhibitory properties of CNS myelin have been demonstrated in a number of different laboratories by a wide variety of techniques, including plating neurons on myelin substrates or cryostat sections of  
15 white matter, and observations of axon contact with mature oligodendrocytes. Therefore, it is well documented that adult neurons cannot extend neurites over CNS myelin in vitro. It has also been well documented that removing myelin in vivo improves the success of regenerative growth over the native terrain of the  
20 CNS. Regeneration occurs after irradiation of newborn rats, a procedure that kills oligodendrocytes and prevents the appearance of myelin proteins. After such a procedure in rats and combined with a corticospinal tract lesion, some corticospinal axons regrow long distances beyond the lesions. Also, in a chick model  
25 of spinal cord repair, the onset of myelination correlates with a loss of its regenerative ability of cut axons. The removal of myelin with anti-galactocerebroside and complement in the embryonic chick spinal cord extends the permissive period for axonal regeneration. Known inhibitory molecules in myelin  
30 include myelin associated glycoprotein (MAG), tenascin-R (TN-R), arretin, and chondroitin-sulphate proteoglycans (CSPGs). Recently, three groups reported the identification in rats and humans of a gene, Nogo, which encodes an inhibitory myelin protein (GrandPre et al, 2000; Prinjha et al, 2000; Chen et al,  
35 2000). Immunization against myelin has been found to allow



extensive axon regeneration after injury, - this demonstrates the enormous potential value of overcoming myelin inhibition. These experiments demonstrate a good correlation between inhibitory factors in myelin and the failure of axons to regenerate in the  
5 CNS.

#### **Multiple Sclerosis**

MS is a degenerative central nervous system disorder involving decreased nerve function associated with the formation of scars  
10 on the insulating sheath known as myelin around nerve cells.

The cause of MS is not known. However, many researchers believe it may be an autoimmune disease, perhaps triggered by a viral infection. There is no definitive clinical test for the  
15 diagnosis of MS. However, an MRI (Magnetic Resonance Imaging) can show areas in the brain where myelin has been damaged.

MS affects approximately 250,000 - 300,000 people in the US. It predominantly afflicts women, Caucasians, and people from  
20 temperate climates. Generally, the onset of MS is diagnosed in people ages 20 - 40.

It is now well accepted that MS lesions contain substantial numbers of premyelinating oligodendrocytes, indicating that:  
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- The potential for repair is not limited by the loss of these cells;
- Interactions between oligodendrocytes and their surrounding environment may determine the outcome of the repair process.

#### **FIRST ASPECT OF THE INVENTION**

  
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##### **Background to First Aspect of the Invention**

Nogo-A has been extensively studied in the context of CNS regeneration and is a pivotal factor in the inhibition of axonal

regeneration after injury (Woolf, 2003). The three major molecules responsible for the growth inhibitory property of CNS myelin - Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) appear to bind to the same glycosylphosphatidyl inositol (GPI)-anchored Nogo-66 receptor (NgR) on the surface of axons (Woolf, 2003). Understanding of the molecular interactions involved in inhibiting neuronal regeneration has led to the exciting possibility that the CNS environment can be manipulated to enhance neuronal regeneration. Apart from these findings, however, other paradigms for Nogo-A action in the CNS have yet to be determined. For example, the localization of the presence of Nogo-A at synapses (Wang et al, 2002) is suggestive of a possible role in modulating synaptic plasticity.

Nogo is expressed in three isoforms, Nogo-A, B and C, all of which share the same C-terminus with two transmembrane domains and an extracellular 66 amino acid loop (Nogo-66) (GrandPre et al, 2000; Fournier et al, 2001). Nogo-A has a large cytoplasmic N-terminal domain (Nogo-N) not found in Nogo-B or Nogo-C (GrandPre et al, 2000; Prinjha et al, 2000; Brittis et al, 2001). Nogo-66 and Nogo-N of Nogo-A have independent inhibitory activity (Fournier et al, 2001; GrandPre et al, 2000). The Nogo-66 loop on the oligodendrocyte surface binds to the Nogo-66 receptor (NgR), which mediates axonal growth retardation (Fournier et al, 2001). In contrast, no receptor or interacting protein for Nogo-N has been recognized so far. Nogo-A is expressed in the brain of the early embryonic CNS, but there is little or no detectable NgR expression in early embryonic neurons (Wang et al, 2002). Nogo-A therefore may have NgR independent functions during embryonic stages. Neuronal regeneration phenotypes in the absence of Nogo-A in knockout mice were not particularly clear, and the exact role of Nogo-A in the CNS remains to be further investigated (Woolf, 2003). In the adult, although Nogo-A has been localized to oligodendrocytes and NgR to mature axons, the exact distribution pattern and relationship between these two molecules along

myelinated axons have not been defined in detail. Knowledge of Nogo-A and NgR distribution at the interface between neurons and oligodendrocytes will be important in understanding their roles in CNS development.

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The establishment and maintenance of the molecular architecture of axonal domains is critical to ensure rapid saltatory conduction of nerve impulses (Pedraza et al, 2001). During myelination, there is a complex, yet precise and efficient, process that ensures the clustering of specific ion channels and other protein molecules to distinct segments along the axon (Dupree et al, 1999; Rasband et al, 2000; 2001a). The Nodes of Ranvier are enriched in Na<sup>+</sup> channels whilst K<sup>+</sup> channels are excluded from this location and instead occupy juxtaparanodal regions. However, during the early stages of both developmental myelination and remyelination, K<sup>+</sup> channel clusters are transiently located at the paranodal between the nodes and juxtaparanodes region (Rasband et al, 1998; Vabnick et al, 1999). This is where the glial cytoplasmic loops come into contact with the axolemma. Adhesion molecules, such as F3/Contactin, neurofascin 155, and Caspr (Paranodin), are located at the paranodes (Einheber et al, 1997; Menegoz et al, 1997; Tait et al, 2000; Kazarinova-Noyes et al, 2001). Studies on dysmyelinating mouse mutants deficient in myelin-related and axonal proteins, such as ceramide galactosyl transferase (CGT) (Ishibashi et al, 2002; Popko B, 2000), F3/Contactin (Boyle et al, 2001), or Caspr (Bhat et al, 2001), have shown that clustering of axonal domain constituents and the exact localization of ion channels, particularly K<sup>+</sup> channels, are dependent on communication between axons and oligodendroglia. However, the molecular mechanisms that regulate the accumulation of K<sup>+</sup> channels into compact zones are not entirely clear.

Contactin-associated protein (Caspr) is a transmembrane protein with an extracellular domain that contains a series of laminin G-

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like domains and EGF repeats (Peles et al, 1997), as well as a cytoplasmic segment with potential binding sites for SH3-containing proteins and 4.1 family proteins (Gollan et al, 2002). Caspr exists in a complex with F3/Contactin, the GPI-anchored molecule (Peles et al, 1997). During myelination, the interaction of Caspr with F3/Contactin is required for the proper transport of Caspr and Na<sup>+</sup> channels to the cell surface (Faivre-Sarrailh et al, 2000; Kazarinova-Noyes et al, 2001). Moreover, this complex constitutes an essential scaffold to maintain the architecture of the axoglial apparatus (Bhat et al, 2001; Boyle et al, 2001). Although neurofascin 155 (NF155) interacts in trans with the Caspr/F3 complex (Charles et al, 2002), the functional consequence of this interaction is still unclear. Judging from Caspr's multiple domain structure, there may exist other glial components that interact with this molecule during myelination.

#### Summary of the First Aspect of the Invention

The inventor explored whether Nogo-A could have a role in the axoglial junction during the period of myelination - in particular, if Nogo-A participates in molecular interactions between axons and oligodendrocytes. He has determined that Nogo-A can be found localized specifically to paranodes, where it interacts with the paranodal junction protein, Caspr. The inventor has also determined that both Nogo-A and Caspr associate with the voltage-gated K<sup>+</sup> channel Kv1.1. Furthermore, the co-localization patterns of Nogo-A/Kv1.1 and Caspr/Kv1.1 during development are closely related. The inventor therefore believes that the interaction of Nogo-A with Caspr plays a role in regulating the location of K<sup>+</sup> channels along the axon during the early period of myelination.

Specifically, the work carried out by the inventor has shown that oligodendrocyte Nogo-A is clustered at specific axoglial junctions, where it interacts directly via its extracellular

Nogo-66 loop with axonal Caspr, and indirectly with K<sup>+</sup> channel proteins. This represents the first NgR-independent Nogo-66 interaction described to date, and has significant implications for the role of Nogo-A in the formation and maintenance of axoglial junction architecture.

Thus, at its most general, the present invention provides materials and methods arising from the determination that Nogo-A and Caspr interact and play a role in myelination. This has important implications particularly in the field of spinal cord injury or other diseases that result in damage to the myelin sheaths.

The invention provides a composition comprising Nogo and Caspr, or mimetics thereof, or a substance capable of promoting interaction between Nogo and Caspr, in combination with a suitable carrier.

Preferably the composition comprises a complex between Nogo and Caspr, or a mimetic of said complex.

The Nogo molecule present in the composition is preferably Nogo-A or a portion or domain thereof. Preferably it comprises Nogo-66, which is found in all three known isoforms of Nogo (A, B and C). It may further comprise other domains of those isoforms; alternatively the Nogo-66 domain may be present in the absence of further portions of Nogo proteins.

The Caspr molecule of the composition is preferably Caspr1 or a portion or domain thereof.

A substance capable of promoting interaction between Nogo and Caspr may be of any molecular type, including, but not limited to a protein, peptide, or small molecule.

- Typically, the substance capable of promoting such interaction will bind to one or both of Nogo and Caspr. For example, it may bind to both proteins, e.g. at the interface between Nogo and
- 5 Caspr. Alternatively it may bind to only one of Nogo and Caspr, possibly stabilising the conformation of that protein in the complex and so promoting association and/or inhibiting dissociation of the complex.
- 10 As an example, the substance capable of promoting interaction between Nogo and Caspr may be an antibody, such as an antibody capable of binding to both Nogo and Caspr, e.g. a bispecific antibody.
- 15 The composition may be a pharmaceutical composition, in which case the carrier will be of a pharmaceutically acceptable type. Preferably the pharmaceutical composition is formulated for injection in vivo, more preferably for injection directly into the CNS. Specifically, there is provided a pharmaceutical
- 20 composition comprising Nogo-A and Caspr.

The invention further provides a composition as described above for use in a method of medical treatment, and particularly for use in the treatment of injury to, or disease of, the CNS, such

25 as spinal cord injury (SCI), multiple sclerosis (MS), epilepsy or stroke.

The invention further provides the use of Nogo in the preparation of a medicament for the treatment of injury to, or disease of,

30 the CNS, wherein the medicament is for administration in combination with Caspr or a mimetic thereof.

Likewise, the invention provides the use of Caspr in the preparation of a medicament for the treatment of injury to, or

disease of, the CNS, wherein the medicament is for administration in combination with Nogo or a mimetic thereof.

Thus the medicament may comprise both Nogo and Caspr or mimetics thereof (i.e. it may be a pharmaceutical composition as described above). The invention accordingly provides a method of manufacturing a pharmaceutical composition comprising admixing Nogo and Caspr or mimetics thereof, with a pharmaceutically acceptable carrier. Alternatively the two components may be administered separately.

Also provided is the use of a substance capable of promoting interaction between Nogo and Caspr, as herein described, in the preparation of a medicament for the treatment of injury or disease to the CNS.

The invention also provides a method of stimulating myelination of a neuron, specifically a neural axon, comprising contacting a neuron or an oligodendrocyte with a composition as described above. This may be performed in vivo, e.g. as a therapeutic method as elsewhere described in this specification, or in vitro.

Also provided is a method of treating a subject having disease of or injury to the central nervous system, comprising administering to the subject one or more pharmaceutical compositions comprising Nogo and Caspr as described above. Specifically, there is provided a method of treating a patient with disease or injury to the CNS, e.g. SCI, MS, epilepsy or stroke, comprising administering to the patient a complex comprising Nogo-A and Caspr.

All therapeutic methods described are considered particularly appropriate for the treatment of spinal cord injury (SCI), multiple sclerosis (MS), epilepsy or stroke.

The invention further provides a method of screening for a substance capable of modulating (preferably promoting) interaction between Nogo and Caspr, the method comprising  
5 contacting Nogo and Caspr with a candidate substance, and determining the interaction between Nogo and Caspr.

The method may further comprise contacting Nogo and Caspr in the absence of said candidate substance under otherwise analogous  
10 conditions, and determining the interaction between Nogo and Caspr.

Preferably the method comprises contacting a complex between Nogo and Caspr with the candidate substance; the complex is preferably  
15 formed before it is contacted with the candidate substance.

The method may be performed by any appropriate method. The skilled person will be well aware of many suitable assay formats, and will be well capable of designing a suitable protocol.  
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One or both of Nogo and Caspr may be present in, or on, a cell. The gene from which the protein is expressed may be endogenous to the cell in question, or it may be present on a vector introduced into the cell. The protein is preferably expressed on the  
25 surface of the cell.

Additionally or alternatively, one or both of Nogo and Caspr may be immobilised on a solid support. One or both may comprise a detectable label as described in more detail below.  
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The invention further provides a method of manufacturing a pharmaceutical formulation comprising, having identified a substance capable of modulating interaction between Nogo and Caspr by a screening method described herein, the further step of  
35 formulating said substance with a pharmaceutically acceptable



carrier. The method may comprise the further step of optimising said identified substance for administration in vivo prior to formulation.

## 5 Cells

The term oligodendrocyte is used herein to refer to oligodendroglial cells capable of laying down a myelin sheath around a neuronal axon in the central nervous system (CNS).

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## Protein sequences

The term "Nogo" is used to encompass all isoforms of the Nogo protein, including Nogo-A, B and C, as well as portions and  
15 isolated domains thereof, including the Nogo-66 domain, as well as mutants and variants thereof having greater than 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to the sequences given below. Orthologous proteins from other mammalian species are also included. Preferably the Nogo protein has the ability to  
20 bind to a Caspr protein, particularly Caspr-1. Nogo-A is particularly preferred.

The term "Caspr" is used to encompass all isoforms of the Nogo protein, including Caspr-1, 2, 3 and 4. Caspr-1 is particularly  
25 preferred. The term is also intended to encompass isolated domains of such Caspr proteins such as the extracellular domain, as well as mutants and variants thereof having greater than 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to the sequences given below. Orthologous proteins from other mammalian species  
30 are also included. The Caspr protein preferably has the ability to bind to a Nogo protein, particularly Nogo-A. It may also have the ability to bind to at least one subunit of a voltage-gated potassium channel, in particular Kv1.1 and/or Kv1.2.

The amino acid sequences of Nogo and Caspr proteins are shown below, along with their GenBank accession numbers;

**Nogo-A** gi:9408096 (CAB99248)

5

1 medldqsplv sssdspprpq pafkyqfvre pedeeeeeee eeedededle elevlerkpa  
 61 aglsaapvpt apaagaplmd fgndfvppap rgplpaappv aperqpswdp spvsstvpap  
 121 splsaaavsp sklpeddepp arppppppas vspqaepvwt ppapapaapp stpaapkrrg  
 181 ssgsvdetlf alpaasepvi rssaenmdlk eqpgntisag qedfsvlle taaslpplsp  
 10 241 lsaasfkehe ylgnlstvlp tegtlqenvs easkevseka ktllidrdlt efseleysem  
 301 gssfsvspka esavivanpr eeiivknkde eeklvsnnil hnqqelptal tklvkedevv  
 361 ssekakdsfn ekrvaveapm reeyadfkpf ervwevkdsk edsdmllaagg kiesnleskv  
 421 dkkcfadsle qtnhekdses snddtsfpst pegikdrpga yitcapfnpa atesiatnif  
 481 pllgdptsen ktdekkieek kaqivteknt stktsnpflv aaqdsetdyv ttdnltkvte  
 15 541 evvanmpegl tpdlvqeace selnevtgtk iayetkmdlv qtsevmqesl ypaaqlcpsf  
 601 eeseatpspv lpdivmeapl nsavpsagas viqpssspse assvnyesik hepenpppye  
 661 eamsvsllkv sgikeeikep eninaalqet eapyisiacd liketklxae papdfsdyse  
 721 makveqvpdp hselvedssp dsepvdlsd dsipdvpgkq detvmlvkes ltetsfesmi  
 781 eyenkeklsa lppeggkpyl esfklsldnt kdtllpdevs tskkekipl qmeelstavy  
 20 841 snddlfsike aqiretetfs dsspieiide fptlissktd sfsklareyt dlevshkse  
 901 anapdgagsl pctelphdls lknigpkvee kisfsddfsk ngsatskvll lppdvvalat  
 961 qaeiesivkp kvlvkeaeek lpsdtekedr spsaifsacl sktsvvdllly wrdikktgkv  
 1021 fgasflfllls ltvfsivsvt ayialallsv tisfryikgv iqaiqksdeg hprfaylese  
 1081 vaiseelvgk ynsalghvn ctikelrrlf lvddldvslk favlmwvfty vgalfngltl  
 25 1141 lllalislfs vpvierhq qidhyglan knvkdamaki qakipglkrk ae

**Nogo-B** gi:9408098 (CAB99249)

30

1 medldqsplv sssdspprpq pafkyqfvre pedeeeeeee eeedededle elevlerkpa  
 61 aglsaapvpt apaagaplmd fgndfvppap rgplpaappv aperqpswdp spvsstvpap  
 121 splsaaavsp sklpeddepp arppppppas vspqaepvwt ppapapaapp stpaapkrrg  
 181 ssgsvvvdll ywrdikktgv vfgasflflll sltvfsivsv tayialalls vtisfryikg  
 241 viqaiqksde ghpfrayles evaiseelvq kysnsalghv nctikelrrl flvddldvsl  
 35 301 kfavlmwvft yvgalfnglt llilalislfs svpvierhq aqidhyglan knvkdamak  
 361 iqakipglkr kae

**Nogo-C** gi:9408100 (CAB99250)

40

1 mdgqkknwkd kvvdlllywrd ikktgvvfga slflllsltv fsivsvtai alallsvtis  
 61 fryikgviqa iqksdegphf raylesevai seelvqkysn salghvncti kelrrlflvd  
 121 dlvdslkfav lmwvftyvga lfngltllil alislfsvpv iyerhqaqid hylglanknv  
 181 kdamakiqak ipglkrkae

**Caspr1** gi:4505463 (NP003623)

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1 mmhlrlfcil laavsgaegw gyygcdeelv gpilyarslga ssyyslltap rfarlhgisg  
 61 wsprigdpnp wlqidlmkhh riravatqgs fnsdwvtry mlygdrvds wtpfyqrghn  
 121 stffgnvnes avvrhdhlfh ftaryirivp lawnprgkig lrlglygcpy kadilyfdgd  
 181 daisyrfrpg vsrslwdvfa fsfkteekdg lllhaegaqg dyvtlelega hlllhmslgs  
 241 spiqprpght tvsaggvlnq qhwhyvrldr fgrdvntld gyvqrfling dferlnldte  
 301 mfigglvgaa rknlayrhnf rgcienvifn rvniadlavr rhsritfegk vafrcldpvp  
 55 361 hpinfggphn fvqvpfgfrr grlavsfrr twdltglllf srlgdglghv eltlsegqvn

421 vsiaqsggrkk lqfaagyrln dgfwhevnfv aqenhavisi ddvegaeerv syplliirtgt  
 481 syffggcpgk asrwdchsnq tafhgcmell kvdgqlvnlt lvegrrlgfy aevlfdtcgi  
 541 tdrscspnmce hdgrcyqswd dficyceltg ykgetchtpl ykesceayrl sgktsgnfti  
 601 dpdgsqplkp fvvycdiren rawtvvrhrd lwtrrtvgss merpflgaiq ywnasweevs  
 5 661 alanasqhce qwiefscyns rllntaggyyp ysfwigrnee qhfywggsqp giqrcacgld  
 721 rscvdpalyc ncdadqpqrw tdkglltfvd hlpvtqvvig dtnrstseaq fflrplracyg  
 781 drnswntisf htgaalrfpp iranhsldvs fyfrtsapsg vflenmggpy cqwrrpyvrv  
 841 elntsrdivvf afdvngngden ltvhsddfef nddewhlvra einvkqarl vdhrpwlrv  
 901 mplqtiwme ydqplyvgsa elkrrpfvgc lramrlngvt lnlegranasegtspnctgh  
 10 961 cahprlpcfh ggrcveryys ytcddcltaf dgpycnhdig gffepgtwmr ynlqsalrsa  
 1021 arefshmlsr pvpgyepgyi pgydtpgyvp gyhgpgyrlp dyprpgrpvp gyrgpvynvt  
 1081 geevsfsfst ssapavlllyv ssfvrzymav likddgtlql ryqlgtspyv yqlttrpvt  
 1141 gqphsinitr vyrlfiqvdy yfplteqkfs llvdsqldsp kalylgrvme tgvidpeiqr  
 1201 yntpgfsgcl sgvrfnvnap lkthfrtprp mtaelaearl vqgelsesnc gamprlvse  
 15 1261 ppeldpwyly pdfpyyhdeg wvailglglv aflllglvgm lylfylvqnr ykgsyhtnep  
 1321 kaaheyhpgs kpplptsgpa qvptptaapn qapasapapa ptpapapgpr dqnlpqilee  
 1381 srse

20 Nogo-66 extends from amino acids 823 to 888 of Nogo-A and has the following sequence:

#### Nogo-66

25 RIYKGVIIQ AIQKSDEGHP FRAYLESEVA ISEELVQKYS NSALGHVNCT IKELRRLFLV DLVDSLK

#### Assay methods

30 As described above, the skilled person is well aware of numerous assay formats which may be appropriate for determining interaction between Nogo and Caspr, and identifying substances which modulate, preferably promote, such interaction.

35 For example, interaction between the two proteins may be studied in vitro by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels, especially for petidyl substances, include <sup>35</sup>S-methionine which may be incorporated into

40 recombinantly produced peptides and polypeptides. Alternatively the complex formed on the solid support may be detected by labelling with an antibody directed against an epitope present on the protein which is not immobilised on the solid support. If no

suitable antibody is available, a recombinantly-produced peptide or polypeptide may be expressed as a fusion protein containing an epitope against which a suitable antibody is available.

5 The protein which is immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known per se. A preferred in vitro interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be  
10 immobilized on glutathione agarose beads. In an in vitro assay format of the type described above a test compound can be assayed by determining its ability to affect the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the  
15 glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

20 An assay according to the present invention may also take the form of a cell-based assay in which at least one of the two proteins is expressed by, preferably on the surface of, a suitable cell. The assay may utilise a cell line, such as a  
25 yeast strain or mammalian cell line, in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

Modulators of Nogo-Caspr interaction identified by the methods  
30 described may be further modified to increase their suitability for in vivo administration.

#### Formulations

- The compositions of the invention may be prepared as pharmaceutical formulations comprising at least one active compound, as defined above, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, including, but not limited to, pharmaceutically acceptable carriers, adjuvants, excipients, buffers, preservatives and stabilisers. The formulation may further comprise other active agents.
- 10 Thus, the present invention further provides a method of making a pharmaceutical composition as previously defined, the method comprising admixing at least one active agent as described herein together with one or more pharmaceutically acceptable ingredients well known to those skilled in the art, e.g., carriers, 15 adjuvants, excipients, etc..

- The term "pharmaceutically acceptable" as used herein pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are, within the scope of sound medical judgment, 20 suitable for use in contact with the tissues of the subject in question (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, adjuvant, excipient, etc. must also be "acceptable" in the sense of being 25 compatible with the other ingredients of the formulation.

- Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, 30 Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

- Formulations may suitably be injectable formulations, e.g. in the form of aqueous, isotonic, pyrogen-free, sterile solutions, in 35 which the active compound is dissolved. Such liquids may

additional contain other pharmaceutically acceptable ingredients, such as anti-oxidants, buffers, preservatives, stabilisers, bacteriostats, suspending agents, thickening agents, and solutes which render the formulation isotonic with the blood or cerebrospinal fluid. Examples of suitable isotonic carriers for use in such formulations include Sodium Chloride Injection, Ringer's Solution, or Lactated Ringer's Injection. Typically, the concentration of the active compound in the liquid is from about 1 ng/ml to about 10 µg/ml, for example from about 10 ng/ml to about 1 µg/ml. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.

#### Administration

Administration of the compositions of the invention will generally be by injection, preferably directly into the CNS. Injection may be directly into the site of damage. Alternatively, injection may be into the cerebro-spinal fluid, typically near the site of disease or injury.

#### Sequence identity

Percent (%) amino acid sequence identity with respect to a reference sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. % identity values may be determined by WU-BLAST-2 (Altschul et al.,

Methods in Enzymology, 266:460-480 (1996)). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. A % amino acid sequence identity value is determined by the number of matching identical residues as determined by WU-BLAST-2, divided by the total number of residues of the reference sequence (gaps introduced by WU-BLAST-2 into the reference sequence to maximize the alignment score being ignored), multiplied by 100.

Percent (%) amino acid similarity is defined in the same way as identity, with the exception that residues scoring a positive value in the BLOSUM62 matrix are counted. Thus, residues which are non-identical but which have similar properties (e.g. as a result of conservative substitutions) are also counted.

In a similar manner, percent (%) nucleic acid sequence identity with respect to a reference nucleic acid is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the reference nucleic acid sequence. The identity values used herein may be generated by the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

#### The subject

The subject to which the compositions and/or treatments of the invention will be administered will be a mammal, preferably an experimental animal such as a rodent (e.g. a rabbit, rat or mouse), dog, cat, monkey or ape, or a farm animal such as a cow, horse, sheep, pig or goat. More preferably, the subject is human.

Generally, the subject will have CNS damage, caused by disease or injury, e.g. a head injury. More preferably, however, the damage is to the spinal cord, e.g. SCI. In experimental animals, the  
5 damage may be experimental. The CNS damage may also result from a disease or disorder, e.g. stroke, epilepsy or a neurodegenerative condition, learning memory-related condition and/or dementia such as Alzheimer's disease or Parkinson's disease.

10

The treatments of the invention will generally be intended for use in conjunction with other therapies, such as surgery and/or rehabilitation.

#### 15 Mimetics

Non-peptide "small molecules" are often preferred to peptides or polypeptides for in vivo pharmaceutical use. Accordingly, mimetics of Caspr and/or Nogo may be designed, especially for  
20 pharmaceutical use. Typically a Nogo mimetic of the present invention will be capable of binding to a Caspr molecule to mimic the effects of Nogo binding to that molecule. Likewise a Caspr mimetic will be capable of binding to a Nogo molecule to mimic the effects of Caspr binding to that molecule.

25

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to  
30 synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large  
35 number of molecules for a target property.



There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or  
5 important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. Alanine scans of peptide are commonly used to refine such peptide motifs. These parts or residues constituting  
10 the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled according to its physical properties, eg stereochemistry,  
15 bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling  
20 process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change  
25 conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template  
30 molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is  
35 peptide based, further stability can be achieved by cyclising the

peptide, increasing its rigidity. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for in vivo or clinical testing.

In the present case, peptide mapping studies may be used to identify the minimal portion of one protein required to interact with the other. This peptide may then be used as a lead compound for mimetic design, as described above.

### Antibodies

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding substance having an binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')<sub>2</sub> fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL

domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers  
5 (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

Diabodies are multimers of polypeptides, each polypeptide  
10 comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (eg by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding  
15 sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be  
20 conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. Current Opinion Biotechnol. 4, 446-449 (1993)), eg prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv  
25 dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in Traunecker et al, Embo  
30 Journal, 10, 3655-3659, (1991).

It may be desirable to "humanise" non-human (eg murine) antibodies to provide antibodies having the antigen binding properties of the non-human antibody, while minimising the  
35 immunogenic response of the antibodies, eg when they are used in

human therapy. Thus, humanised antibodies comprise framework regions derived from human immunoglobulins (acceptor antibody) in which residues from one or more complementary determining regions (CDR's) are replaced by residues from CDR's of a non-human species (donor antibody) such as mouse, rat or rabbit antibody having the desired properties, eg specificity, affinity or capacity. Some of the framework residues of the human antibody may also be replaced by corresponding non-human residues, or by residues not present in either donor or acceptor antibodies. These modifications are made to the further refine and optimise the properties of the antibody.

Specific embodiments of the first aspect of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

**Brief Description of the Figures relating to the First Aspect of the Invention**

**Figure 1.** Glia-derived Nogo-A clusters at the paranodes in CNS. A. Adult rat brain stem sections were double immunolabeled for Nogo-A (green; a, c, d, f, g, and i) and the Kv1.1 K<sup>+</sup> channel  $\alpha$ -subunit (red; b, c, h, and i) or PAN Na<sup>+</sup> channel (red; e and f). For negative control, Nogo-A antiserum (1:200) was premixed with antigen before staining of an adult brain stem section (j). Two Nogo-A antibodies, one developed in the inventor's lab (a-f) and another was developed in Dr. Stritmatter's lab (g-i), were used in this study. c, f and i are merged images of a-b, d-e and g-h, respectively. B. Ultrastructural localization of Nogo-A at the paranodes in rat spinal cord. (a) Immunogold labeling of cross sections of myelinated axons revealed that the gold particles were detected at the inner and outer myelin sheaths. (b and c) Immunogold

- particles of Nogo-A are found within glial loops and the compacted myelin. (d and e) In longitudinal sections of paranodes, immunogold particles of Nogo-A located at the tips of glial loops in the axoglial junction and some within the axon in d. A higher magnification of the boxed areas of d and e were shown in d' and e'. ax: axon; OL: oligodendrocyte. Gold particles are indicated with arrows. Bars: 5  $\mu$ m for Aa-i, 10  $\mu$ m for Aj, 200 nm for Ba, d and e, 100 nm for Bb, c, d' and e'.
- Figure 2.** Expression and localization of Nogo-A in the CNS myelinated axons from EAE rats and CGT<sup>-/-</sup> mice. A and B. Spinal cord sections from EAE rats were double immunostained for Nogo-A (green, A), Caspr (green, B) and Kv1.1 (red, A and B). The star marks a Nogo-A positive cell body, and the arrowhead indicates an undisrupted paranodal Nogo-A labeling. C. Numbers of Nogo-A and Caspr clusters in several microscopic fields of both normal and EAE rat spinal cord sections were counted. Values are given as mean  $\pm$  SEM from at least 3 independent experiments. Nogo-A clusters were dramatically reduced in EAE compared to normal rats. The double asterisks (\*\*) indicates a significance level of  $P < 0.01$ . D. Western blotting result showing that Nogo-A was significantly downregulated in spinal cord of EAE animals, but Caspr expression was only affected slightly. The single asterisk represents a significance level of  $P < 0.05$ . E, F, G, and H. Spinal cord sections from wild type and CGT<sup>-/-</sup> mice were double-labeled for Nogo-A (green) and PAN Na<sup>+</sup> channel. In wild-type (P16), Nogo-A clusters at paranodes and PAN Na<sup>+</sup> channels congregated at the nodes of Ranvier (E). In CGT<sup>-/-</sup> mice (P16), Nogo-A segregation at paranodes and PAN Na<sup>+</sup> channel clustering were hardly detected (F). Nogo-A labeling appears to be loosely spiraled around the axon in CGT<sup>-/-</sup> mice (P21; H), but clusters specifically into the paranodal region in wild type mice (P21; G). Bars: 10  $\mu$ m for A-B and 5  $\mu$ m for E-H. I and J. Immunogold labeling of Nogo-A in longitudinal sections of paranodes from P16 CGT<sup>-/-</sup> mice spinal cord demonstrated that gold

particles were visible in the abnormally reversed loops. Panels I' and J' are high magnification of the boxed areas in I and J, respectively. Arrows indicate the gold particles. Stars indicate the reversed paranodal loops. ax: axon. OL: oligodendrocyte.

5 Bars: 200 nm for I and J, 50 nm for I' and J'.

**Figure 3. Caspr associates with Nogo-A *in vitro*.**

A. NgR distributes diffusely along the myelinated axon. (a) Tissue lysates from various regions of the CNS of adult rats were  
10 subjected to Western blot using antibodies against NgR and  $\gamma$ -tubulin. (b) Brainstems from postnatal day 1-30 (P1-P30) rats were subjected to Western blot using antibodies against NgR and  $\gamma$ -tubulin. Adult hippocampus (c-e) and brainstem (f-h) sections were double stained for NgR (green, c and f), MAP2 (red, d) and  
15 Kv1.1 (red, g). e and h represent merged images of c, d, and f, g, respectively. Scale bars for c to h: 10 $\mu$ m.

B. Nogo-A associates with Caspr/F3. (a) Detergent lysates of brain membrane fractions from adult mice were immunoprecipitated with Caspr, Nogo-A, and NB3 antibodies as well as non-immune IgG.  
20 The immunoprecipitates and detergent extracts from brain (Brain) together with the protein-A beads (Beads) were subjected to Western blot using antibodies against Caspr, Nogo-A, F3, and NB3. (b) Membrane fractions of Nogo-A/Caspr/F3-, Nogo-A/F3- and Nogo-A- transfected CHO cells were immunoprecipitated with antibodies  
25 to Caspr and Nogo-A as well as non-immune IgG. The immunoprecipitates and brain extracts (Brain) were subjected to Western blot analysis using antibodies against Nogo-A, Caspr and F3. (c) Detergent lysates of membrane fractions from P15 rat cerebral cortex were loaded onto a linear sucrose gradient.  
30 Twelve gradient fractions were collected, subjected to SDS-PAGE and analyzed for the distribution of Nogo-A, Caspr and F3 by Western blotting. Fraction 1 is the lowest density fraction recovered from the top of gradient. The last lane that is labeled "total" indicates the levels in homogenates before loading onto  
35 the sucrose gradient.

**Figure 4.** Caspr expressing cells adhere to Nogo-66.

A to L. Caspr/F3- (A, E and I), F3- (B, F and J), and mock- (C, G and K), as well as PI-PLC treated Caspr/F3- (D, H and L) CHO cells were plated onto substrates coated with Nogo-66 peptide, recombinant GST-Nogo-66 protein, and GST, respectively. Scale bar: 8  $\mu$ m. M to N. Quantification of cells adherence to various substrates. Caspr/F3- CHO cells (in the presence or absence of PI-PLC treatment), but neither F3- nor wild type CHO cells, bound to Nogo-66 peptide and GST-Nogo-66. Bars represent the number of adherent cells (expressed as mean  $\pm$  SEM) from at least 3 independent experiments (M). O. At the end of cell adhesion assay after PI-PLC treatment, Caspr/F3-CHO and F3-CHO cells and their culture supernatant were collected, and subjected to Western blotting analysis after normalizing for total protein to detect F3/Contactin. 1, mouse brain; 2, F3-CHO cell lysate; 3, Caspr/F3-CHO cell lysate after PI-PLC treatment; 4, Caspr/F3-CHO cell medium after PI-PLC treatment. The asterisk (\*) indicate a significance level of  $P < 0.05$ . Caspr/F3- CHO cells adhered to Nogo-66 and GST-Nogo-66 with equal efficiency after treatment with increasing concentrations of PI-PLC (0.02, 0.04 and 0.06 U/ml) (N).

**Figure 5.** The Nogo-A/Caspr complex interacts with  $K^+$  channels.

A. (a) P7 mouse brain membrane extracts were immunoprecipitated with Caspr, Nogo-A, Kv1.1, Kv1.2 and NB3 antibodies as well as non-immune IgG. The indicated immunoprecipitates and brain extracts (Brain) were subjected to Western blot analysis using antibodies against Kv1.1, Kv1.2, Nogo-A and Caspr. (b) GST pull-down assay was performed using recombinant GST-Nogo-66, GST-Nogo-N and GST from adult mouse brain extracts. The indicated precipitates and brain extracts (brain) were probed with Caspr and Kv1.1 antibodies, following SDS-PAGE separation.

- B. (a) Membrane fraction of CHO, F3-CHO, Caspr/F3 CHO cells, as well as brain extracts were immunoblotted using Caspr antibodies, following SDS-PAGE separation. (b) After transient transfection with the Kv1.1 expression construct RBG4/Kv1.1, membrane extracts
- 5 of CHO, F3-CHO, Caspr/F3 CHO cells were incubated with GST-Nogo-66 or GST, respectively. The eluted proteins were separated by SDS-PAGE and probed with Caspr and Kv1.1 antibodies. IP: immunoprecipitation; WB: Western blot.
- 10 **Figure 6.** Immunohistochemical labeling of Nogo-A, Caspr and Kv1.1 at different postnatal days in rat brainstem.
- A. Brainstem sections of P5 to adult rats were double labeled for Caspr (green) and Kv1.1 (red). Scale bar: 5  $\mu$ m. B. Sections from P1 to P30 rats were double labeled for Nogo-A (green) and Kv1.1
- 15 (red). Scale bar (in o): 5  $\mu$ m. C. (a) The lengths of Nogo-A, Kv1.1 immunostaining and their overlap were measured from micrographs ( $\mu$ m, mean  $\pm$  SEM). (b) The number of overlapping Nogo-A/Kv1.1 or Caspr/Kv1.1 clusters at paranodes was counted.
- 20 **Figure 7.** Distribution of Nogo-A, Caspr and Kv1.1 in EAE rats and Shiverer mice.
- A and B. Double immunofluorescence labeling for Caspr (green) and Kv1.1 (red) in brainstem sections of EAE (A) and control (B) rats. The insets represent magnified views of the Kv1.1 labeling.
- 25 Scale bars: 10  $\mu$ m for A-B and 5  $\mu$ m for insets of A-B. C to G. Double labeling for Nogo-A (green) and Kv1.1 (red) in spinal cord sections of wild type (C) and Shiverer (D-G) mice. F is a merged picture of D and E. Arrows in C, F and G indicate Nogo-A positive cell bodies. Bars: 10  $\mu$ m. H and I. Double immunofluorescence
- 30 staining of Caspr (green) and Kv1.1 (red) in brainstem sections of wild-type (H) and Shiverer (I) mice. Bar: 10  $\mu$ m. J. The distances between paired Kv1.1 staining in EAE with control rats and Shiverer (Shi) with wild-type (WT) mice were measured from micrographs ( $\mu$ m, mean  $\pm$  SEM), respectively. The values in EAE



rats or *Shiverer* mice were significantly reduced when compared to their controls, respectively (asterisks \*\* indicates a significance level of  $P < 0.01$ ).

- 5 **Figure 8.** The interaction between Nogo-A and Caspr at the paranodes may play a role during myelination.
- In addition to NF155, the inventors' findings suggest that paranodal Nogo-A is a glial ligand for neuronally expressed Caspr. Paranodal Nogo-A *trans*-interacts with axonal Caspr, and
- 10 may play a role in  $K^+$  channel localization during the early stages of myelination (from P5). With the firm establishment of axoglial junctions in the adults,  $K^+$  channels were excluded from paranodes where Nogo-A/Caspr interaction is maintained, however, the detail mechanism for this separation remains to be explored.
- 15 N: Node of Ranvier, PN: paranode, JPN: juxtaparanode.

#### Detailed Description of the First Aspect of the Invention

##### Results

- 20 **Nogo-A is localized to the paranodes of myelinated axons**
- The distribution of Nogo-A was examined along the white matter tracts of adult rat brainstem. In longitudinal sections, similar localization patterns of Nogo-A were observed with two different
- 25 Nogo-A antibodies: one was developed in the inventor's lab (Fig. 1A, a-f; Liu et al, 2002) and another was a kind gift from Dr. Stephen Stritmatter (Yale University) (Fig. 1A, g-i; Wang et al, 2002). Nogo-A immunoreactivity (green) was confined specifically to paranodal segments along myelinated axons (Fig. 1A), as
- 30 evidenced by double immunofluorescence labeling with Kv1.1 (red; Fig. 1A, a-c, g-i) or the  $Na^+$  channel (red; Fig. 1A, d-f). The specific labeling of Nogo-A in axonal domains was undetectable after the Nogo-A antisera (1:200) were premixed with 100 fold molar excess of antigen (Fig. 1Aj). The Nogo-A staining (green)
- 35 flanks nodal  $Na^+$  channel labeling (red), and is flanked by

juxtaparanodal Kv1.1 labeling (red), thus reflecting its paranodal location. Similar observations were made in other nerve fiber-rich CNS sites such as the corpus callosum and the spinal cord (not shown). In the sections examined, Nogo-A was also  
5 detected in both neurons and oligodendrocytes (not shown), consistent with other published studies (Huber et al, 2002; Wang et al, 2002; Liu et al, 2002). These observations suggest that Nogo-A may be enriched at the paranode and is a component of the paranodal protein complex.

10

The specific paranodal location of Nogo-A was further investigated using immuno-electromicroscopy (IEM). Consistent with previous observations (Huber et al, 2002), Nogo-A immuno-reactivity was high in the inner and outer loops of the myelin  
15 sheath (Fig. 1Ba), and low in the compact myelin of rat spinal cord (Fig. 1Bb). Notably, in longitudinal sections, Nogo-A immunoreactivity was high in the expanded terminal glial loops (Fig. 1Bb and c) and the axoglial junction between the loops and the axolemma (Fig. 1Bd and e) at paranodes, and is present only  
20 occasionally in the paranodal axon (Fig. 1Bd). These observations indicate that Nogo-A is a component of the CNS paranodes.

Nogo-A is a paranodal element mainly derived from oligodendroglia. Nogo-A is predominantly expressed in oligodendroglial cell bodies  
25 and white matter of the adult CNS (Huber et al., 2002). To characterize the cellular origin of the paranodal Nogo-A, we examined Nogo-A's distribution in two animal models: experimental autoimmune encephalomyelitis (EAE), a condition involving progressive CNS demyelination (Swanborg 2001), and CGT<sup>-/-</sup> mice  
30 known to display a presence of reversed lateral loops but an absence of transverse bands, and abnormal localization of K<sup>+</sup> channels along their axons (Dupree et al, 1999). At the peak of demyelination in adult EAE rats, their longitudinal spinal cord sections were prepared for double immunofluorescence staining for  
35 Nogo-A or Caspr, and Kv1.1. The density of Nogo-A positive

paranodal congregates was significantly decreased (by around 90%) in sections from EAE rats (Fig. 2A and C) compared to those from control animals. Only occasional foci of Nogo-A positive paranodal clusters (arrowhead; Fig. 2A) and oligodendroglial immunoreactivity remained (star; Fig. 2A). In correlation with the loss of paranodal staining, Nogo-A expression in the spinal cord was downregulated in EAE rats (Fig. 2D). However, Caspr expression was affected to a much lesser extent by this disorder in the spinal cord (Fig. 2B-D).

In P16 wild-type mice, Nogo-A (green) clusters beside the congregated Na<sup>+</sup> channels (red) at paranodes (Fig. 2E). In P16 CGT<sup>-/-</sup> mice, however, congregation of both Nogo-A (green) and Na<sup>+</sup> channels (red) was hardly detected along the axons (Fig. 2F). Immunofluorescence analysis on spinal cord sections from P21 wild type and CGT<sup>-/-</sup> mice was performed using another axonal marker, the 200 kDa neurofilament, in combination with Nogo-A antibodies. Loose spiral-like labeling of Nogo-A was detected along the neurofilament labeled axon in CGT<sup>-/-</sup> mice (Fig. 2H). This rather deranged labeling pattern is clearly different from the compact clustering pattern of Nogo-A labeling in wild type animals (Fig. 2E and G), revealing that the congregation of the glial Nogo-A along the axon was severely disrupted in the mutants. IEM observations showed that Nogo-A immunoreactivity was clearly present in the reversed lateral loops at paranodes in these mutant mice (Fig. 2I and J). Given widespread demyelination and oligodendroglial damage in EAE rats and abnormal nodal and paranodal structures in CGT<sup>-/-</sup> mice, loss of Nogo-A congregation at the paranodes suggests that paranodal Nogo-A is predominantly associated with OLs.

#### **NgR is uniformly distributed along myelinated axons**

The inventor next investigated the expression and distribution of the Nogo-66 receptor (NgR), to find out if it also exhibits domain specific congregation patterns in myelinated axons.

Immunoblot analyses using NgR antibodies demonstrated that NgR expression is higher in adult brainstem, hippocampus, and cerebral cortex, but is much lower in spinal cord (Fig. 3Aa). NgR is detectable as early as postnatal day 1, with its expression level maintained till postnatal day 14, and subsequently showing a gradual decrease from 3 weeks of age (Fig. 3Ab). To confirm that the NgR antibodies used could label NgR on neurons, the inventor stained rat hippocampal sections and showed that NgR co-localized with the neuron-specific microtubule-associated protein 2 (MAP2) in cell bodies and processes (Fig. 3A, c-e). Double labeling for NgR and Nogo-A is difficult due to the rabbit polyclonal origin of both antibodies. The inventor instead performed double labeling of NgR and Kv1.1. In longitudinal brainstem sections, NgR is uniformly distributed along axons, contrasting with the congregated K<sup>+</sup> channel labeling (Fig. 3A, f-h). Similar NgR labeling was observed in P1, P5, P14, and P30 age groups (not shown). The observation that the NgR localization pattern is distinctly different from the congregated Nogo-A at the paranode raises the possibility that Nogo-A may interact with an axonal receptor other than NgR in these specific axon-glial domains.

#### **Nogo-A interacts with paranodal Caspr/F3 complex**

Given the paranodal location of Nogo-A, the inventor investigated whether paranodal axonal components, such as Caspr, F3, and NB3 (an F3-related molecule) (Lee et al, 2000), are Nogo-A binding partners. Nogo-A, Caspr, F3, and NB3 were immunoprecipitated from membrane extracts of adult rat brain. Western blot analysis of the immunoprecipitates resolved on SDS-PAGE revealed that Nogo-A, Caspr, and F3, but not NB3, were present in the immunocomplexes pulled down by either Nogo-A or Caspr antibodies (Fig. 3Ba). Immunoprecipitation (IP) studies were also performed on Caspr/F3-, F3-, and wild type CHO cells transiently transfected with a Nogo-A expression construct. Transfection was performed using Caspr/F3 expressing cells because F3 is required for proper cell

surface expression of transfected Caspr (Faivre-Sarrailh et al, 2000). Western blotting demonstrated that Nogo-A and Caspr indeed associate with each other in Caspr/F3-, but not F3- and wild type CHO cells (Fig. 3Bb). These observations suggest that Nogo-A  
5 interacts specifically with Caspr rather than F3.

**The majority of Nogo-A and Caspr/F3 complex are not co-localized in lipid rafts**

F3 and Caspr are associated with lipid rafts in neurons,  
10 transfected CHO cells, and OLs (Buttiglione et al, 1998; Krämer et al, 1999; Faivre-Sarrailh et al, 2000). To investigate the relationship between Nogo-A and the Caspr/F3 associated microdomains during development, rat cerebral cortexes were lysed and fractionated in a sucrose density gradient according to an  
15 established procedure (Krämer et al, 1999). The majority of the known raft-associated protein F3 was detected in fraction 5, and all Caspr in the lysate was virtually found in this fraction, suggesting that both molecules are located in neuronal rafts. On the other hand, the majority of Nogo-A was found in fractions 8-  
20 12 that are enriched in cytoskeleton-associated proteins, although a minor portion was present in fractions 5-7 (Fig. 3Bc). Similar results were obtained using cerebral cortex lysates of both P15 and adult (not shown). These observations demonstrate that the majority of Nogo-A is not present in the neuronal rafts  
25 in vivo, and provides indirect support for the notion that paranodal Nogo-A is derived from the oligodendroglial membrane.

**Nogo-A interacts directly in trans with Caspr via the extracellular Nogo-66 loop**

30 The extracellular domain of Nogo-A consists of a 66 amino acid loop between its two transmembrane domains, known as the Nogo-66 domain (Fournier et al, 2001). Any interaction in trans between Nogo-A and Caspr should involve the Nogo-66 domain. The inventor next investigated whether the Nogo-A and Caspr associate directly  
35 in a trans-manner that is independent of F3 using a cell adhesion

assay. Different CHO cell lines (expressing Caspr or otherwise) were plated onto substrates coated with the Nogo-66 peptide or recombinant GST fusion proteins containing Nogo-66 (GST-Nogo-66) or the cytoplasmic N-terminal domain of Nogo-A (GST-Nogo-N) as well as GST. Only Caspr/F3-expressing, but neither F3-expressing nor wild type CHO cells, adhered readily to Nogo-66 (Fig. 4A-C) or GST-Nogo-66 (Fig. 4E-G). None of these CHO cell types adhered well to GST (Fig. 4I-K) or GST-Nogo-N (not shown). Quantification of adhering cells indicated that the number of Caspr/F3-CHO cells binding to both Nogo-66 and GST-Nogo-66 was much higher than in other experimental groups (Fig. 4M).

To investigate if Nogo-66 interacts with Caspr only or a binding pocket generated by the cell surface Caspr/F3 complex, the inventor removed the GPI-linked F3 using phosphatidylinositol-specific phospholipase C (PI-PLC). After a PI-PLC treatment, Caspr/F3-CHO cells still adhered to both Nogo-66 and GST-Nogo-66, but not to GST coated substrates (Fig. 4D, H, L and M). To ensure that F3 was completely removed from the cell surface after PI-PLC treatment, increasing concentrations of PI-PLC (0.02, 0.04 and 0.06 units/ml) were used. Cell binding was constant in all three different concentrations of PI-PLC (Fig. 4N). In agreement with previous work (Faivre-Sarrailh et al, 2000), Western blot (Fig. 4O) and immunostaining (not shown) using F3 antibodies demonstrated that a significant amount of F3 was indeed removed from Caspr/F3-CHO cells after treatment with PI-PLC. This lack of effect of PI-PLC on the cell adherence to Nogo-66 substrates suggests that F3 is not directly involved in the *trans*-interaction between Nogo-66 and Caspr.

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#### **The Nogo-A/Caspr complex interacts with K<sup>+</sup> channels**

During myelination, the proper segregation of K<sup>+</sup> channels to juxtaparanodes requires an intact paranodal axoglial junction (Vabnick and Shrager, 1998). This structure is formed and maintained by axonal molecules such as Caspr and F3, as well as

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- by glial specific molecules during myelination and remyelination (Girault and Peles, 2002). The inventor hypothesized that the interaction of Nogo-A with Caspr forms an axo-glial signaling connection that could influence K<sup>+</sup> channel's final location at juxtaparanodes during the early stages of myelination.
- Immunoprecipitation analyses were performed to investigate whether K<sup>+</sup> channels could physically interact with Nogo-A/Caspr complex in the CNS. Both Nogo-A and Caspr antibodies reciprocally precipitated Kv1.1 and Kv1.2, but not Kv2.1 (not shown), from P7 and adult (not shown) mouse brain extracts, while NB3 antibody and non-immune IgG did not (Fig. 5Aa). Kv2.1 is generally found in neuronal cell bodies and proximal dendrites, but is excluded from axons (Trimmer et al, 1991). In GST pull-down assays using membrane extracts of adult mouse brain, both Caspr and Kv1.1 could be pulled down by GST-Nogo-66, but not by GST-Nogo-N or GST itself (Fig. 5Ab). These results support the notion that Nogo-66 is a *trans*-interacting partner of Caspr, and that K<sup>+</sup> channels may interact with the complex.
- Nogo-66 via Caspr interacts indirectly with K<sup>+</sup> channels**
- The inventor next investigated whether Nogo-66 could directly interact with K<sup>+</sup> channels. Kv1.1 cDNA was transiently transfected into Caspr/F3-, F3- and wild type CHO cells and the membrane extracts were subjected to a GST pull-down assay using GST and GST-Nogo-66 fusion proteins, respectively. Wild type and F3-expressing CHO cells did not express Caspr (Fig. 5Ba). Western blot analysis showed that both Kv1.1 and Caspr could only be precipitated by GST-Nogo-66, but not GST, from the Caspr/F3-expressing, but not F3-expressing and wild type CHO cells (Fig. 5Bb). These results demonstrate that Nogo-66 could interact indirectly, at least *in vitro*, with K<sup>+</sup> channels via Caspr.

**Nogo-A and Caspr share a similar spatial and temporal relationship to Kv1.1 along myelinated axons**

In view of the potential interaction between the paranodal Nogo-A/Caspr and Kv1.1 established above, the inventor explored the dynamic relationship between Nogo-A/Caspr and Kv1.1 distribution along myelinated axons during development. Double immunofluorescence labelings of Caspr and Kv1.1, and of Nogo-A and Kv1.1, were performed on brainstem sections of rats at various postnatal days. Congregations of both Caspr and Kv1.1 labeling were apparent from approximately P5 onwards (Fig. 6Aa). From P5-P14 (Fig. 6A, a-c), Caspr staining at paranodes overlapped that of Kv1.1, suggesting co-localization of both molecules at this critical early period of myelination. At P30 (Fig. 6Ad), Kv1.1 labeling became more distinctly juxtaparanodal, with only minimal bands of overlap with Caspr at paranodal-juxtaparanodal borders. In the adults (Fig. 6Ae), Caspr and Kv1.1 were segregated into their different microdomains along the myelinated axons. Double immunofluorescence staining for Nogo-A and Kv1.1 at P1 (Fig. 6B, a-c) revealed that Nogo-A was diffusely labeled along the nerve fibers. At P5 (Fig. 6B, d-f), clustering and aggregation of Nogo-A staining became more evident. However, the staining pattern still did not have well-defined domains or borders. Nodal gaps were apparent and hemi-nodes were seen as well. From P7 (Fig. 6B, g-i), Nogo-A distribution demonstrated an obvious clustering towards the paranodes. From P5-P14 (Fig. 6B, j-l), there were varying degree of overlap between congregates of Nogo-A and Kv1.1 immunostaining at both paranodal and juxtaparanodal regions. At P30 (Fig. 6B, m-o), the Kv1.1 congregates were exclusively localized to juxtaparanodes, akin to the situation in adult animals.

Co-localization of the Nogo-A/Kv1.1 and Caspr/Kv1.1 was quantified by measuring the lengths of Nogo-A and Kv1.1 labeled regions on captured images (Fig. 6Ca). The average length of a Nogo-A labeled region was about 9  $\mu$ m at P5, 5  $\mu$ m at P7, but this shortened to about 2  $\mu$ m from P14 to adult, suggesting that Nogo-A is progressively congregated into narrower bands during the early



stages of myelination. The average length of a Kv1.1 labeled region did not demonstrate such a marked change with time: 6  $\mu$ m at P5 to 8  $\mu$ m in the adult. Of note was the change in terms of the length of overlap between Nogo-A and Kv1.1 labeling: it  
5 decreased from 4  $\mu$ m at P5, 2  $\mu$ m at P14, and 1  $\mu$ m at P30 to approximately 0  $\mu$ m in the adult. This change demonstrates a transient co-localization of Nogo-A and Kv1.1 in paranodal regions before compact myelin is fully laid down. The degree of co-localization between Nogo-A/Kv1.1 and Caspr/Kv1.1 was also  
10 compared (Fig. 6Cb). From P5-P14, >60% of paranodes in every field of view were double labeled for Nogo-A/Kv1.1 and Caspr/Kv1.1, respectively. In adults, Nogo-A and Caspr separated from Kv1.1 and a complete segregation was observed. Given that K<sup>+</sup> channels bind to the Nogo-A/Caspr complex, these observations imply that  
15 the Nogo-A/Caspr complex may transiently interact with K<sup>+</sup> channels, and as such may cooperatively regulate the paranodal localization of Kv1.1 during the early stages of myelination.

#### **Nogo-A and K<sup>+</sup> channel in demyelinating animal models**

20 *Shiverer* is a hypomyelinating mutant mouse that lacks myelin basic protein (MBP) and has axons with normal oligodendroglial ensheathment, but displays aberrant axoglial junctions and abnormal localization of K<sup>+</sup> channels along its axons (Rasband and Trimmer, 2001a). To investigate the roles of glia-related  
25 molecules in the regulation of K<sup>+</sup> channel localization in myelinated axons, the distribution of Nogo-A, Caspr, and Kv1.1 was examined in both EAE rats and *Shiverer* mice. Double immunofluorescence staining demonstrated that both disorganized Caspr (green) and Kv1.1 (red) labelings were still detectable in  
30 the paranodal region of EAE rat (Fig. 7A), compared to normal rat (Fig. 7B) brainstem. In contrast to the location of paranodal Nogo-A (green) and juxtaparanodal Kv1.1 (red) in the spinal cord sections of normal mice (Fig. 7C), Nogo-A staining was diffused along the axons and its congregates were hardly detectable in the  
35 paranodal region of *Shiverer* mice (Fig. 7D-G). Nogo-A

immunoreactivity in OLs, however, remained intact and distinct (arrows; Fig. 7C, F and G). In accordance with previous observations (Poliak et al, 2001), disorganized Caspr and Kv1.1 labeling co-localized at paranodes in *Shiverer* mice (Fig. 7I) but not normal mice (Fig. 7H). Quantitative analysis of the distance between paired Kv1.1 immuno-stainings demonstrated that the distances between the pairs were significantly reduced in both EAE and *Shiverer* mice versus normal animals ( $P < 0.01$ ; Fig. 7J). These observations suggest that, in both pathological conditions of EAE rats and *Shiverer* mice displaying paranodal junction defects,  $K^+$  channels are actually relocated to the paranodes. Concomitantly, the congregation of Nogo-A at the paranode was markedly reduced. Thus, in addition to axonal molecules, certain glia-derived molecules involved in formation of axoglial junctions may also be essential for proper  $K^+$  channel localization at juxtaparanodes in normal adult animals. At the moment, the molecular structural basis for these changes is unknown to the inventor, but may well be related to the interaction between  $K^+$  channels and the Nogo-A/Caspr complex.

#### Discussion relating to the First Aspect of the Invention

Nogo-A, but not the Nogo-66 receptor, is a hallmark of the paranode

The location of Nogo-A in oligodendroglia and CNS myelin has already been described (Huber et al, 2002; Liu et al, 2002; Wang et al, 2002). Specifically, Nogo-A has been localized to oligodendrocyte cell bodies and processes and to the innermost loop and outer loop of the myelin sheath (Huber et al, 2002). In the developing cerebellum, the time course of appearance of Nogo-A mRNA and protein parallel the time frame for myelination, occurring in a period just prior to the expression of myelin basic protein. These observations suggest a role for Nogo-A during myelination.

Increasing evidence points to the importance of axon-glial communication in the regulation of oligodendrocyte differentiation (Barres et al, 1999; Marcus et al, 2000) and ion channel clustering on neurites (Dupree et al, 1999; Ishibashi et al, 2002). The inventor has investigated whether Nogo-A was localized to distinct sites involved in such intercellular signaling and has shown that Nogo-A is mainly localized at the gaps between Na<sup>+</sup> and K<sup>+</sup> channels along axons and its immunoreactivity is clearly located at sites where glial loops make contact with the axonal membrane surface in adult CNS. This suggests that Nogo-A is a paranodal component, which is further confirmed by the observations in several pathological animal models. In accordance with Nogo-A downregulation, its congregates are significantly reduced at paranodes in EAE animals. Nogo-A immunoactivity presents in the reversed paranodal loops in CGT<sup>-/-</sup> mice, however, congregated Nogo-A is also hardly detectable at paranodes in both CGT<sup>-/-</sup> and *Shiverer* mice. Altogether, these observations support the notion that Nogo-A is a paranodal glial component (Fig. 8).

The staining pattern and oligodendroglial origin of paranodal Nogo-A raised the question as to whether it interacts with components on the axonal surface. So far, the only known high affinity neuronal receptor for Nogo-A is the Nogo-66 receptor (NgR). Previous work has shown that NgR expression in neurons and along myelinated axons is predominantly found in adult animals and that its expression during myelination is minimal (Fournier et al, 2001; Hunt et al, 2002; Wang et al, 2002). The inventor's results are consistent with these findings, in that they show that NgR is located in the brainstem on neuronal cell bodies and uniformly distributed along myelinated axons from the early stages of development until adulthood. It is intriguing that there is significant Nogo-A clustering at paranodes, while the NgR distribution pattern remained diffuse along axons. As the congregation of Nogo-A coincides with the developmental period of

myelination, paranodal Nogo-A may therefore participate in this process and may interact with a molecule other than NgR, for a function distinct from inhibition of axonal sprouting.

5   **Paranodal Nogo-A is a glial ligand of Caspr**

At the paranodes, the GPI-anchored axonal F3/contactin exists as a complex with the membrane protein Caspr. The Caspr/F3 complex interacts with NF155, a glial-derived molecule, in *trans*, and is an example of axoglial molecular connection at the paranode  
10 (Girault and Peles, 2002). The inventor has shown that Nogo-A associates specifically with the Caspr/F3 complex, but not NB3, another paranodal molecule, in co-IP assays, implicating an interaction between Nogo-A and the complex *in vivo*. The  
15 observation that the majority of CNS Nogo-A is not co-localized with the complex in neuronal lipid rafts implies that this interaction probably occurs in a *trans*-manner, with Nogo-A from the OL membrane interacting with Caspr/F3 from the axonal membrane. Although it remains a possibility that axonal Nogo-A is a *cis*-binding partner of Caspr/F3 complex, and is dependent upon  
20 myelination for congregation, this seems unlikely since the paranodal Nogo-A is predominantly expressed by OLs in the CNS (Huber et al, 2002; Liu et al, 2002; Wang et al, 2002). To support the notion, the inventor has further shown that Caspr/F3-expressing CHO cells bind to both substrates coated with Nogo-66  
25 peptides and GST-Nogo-66, where the binding must occur in a *trans* manner. That binding occurs even after removal of F3 from the cells via PI-PLC treatment further implies that Nogo-A interacts directly with Caspr (Fig. 8).

30   **Nogo-A may complement Caspr in regulating Kv1.1 location**

There is strong evidence suggesting that K<sup>+</sup> channel accumulation at the juxtaparanode is influenced by myelinating OLs (Vabnick and Shrager, 1998). *Shaker*-type K<sup>+</sup> channels are multi-protein complexes composed of Kv1.1, Kv1.2 and Kvβ2 subunits (Rasband and  
35 Trimmer, 2001b). In the myelinated axons of the CNS, K<sup>+</sup> channel

labeling becomes more prominent during the progression of postnatal development, initially localizing to juxtaparanodes and also to paranodal bands that alternate with Caspr immunoreactivity (Rasband et al, 1999). At later stages of postnatal development, K<sup>+</sup> channels are excluded from the paranodes and become exclusively juxtaparanodal. The inventor has shown that Nogo-A, via Caspr, associates indirectly with Kv1.1. However, the developmental changes in the distribution pattern of Nogo-A and Kv1.1, as well as in those of Caspr and Kv1.1, are similar, implying that the interaction between Kv1.1 and Nogo-A/Caspr complex occurs, at least transiently, when Nogo-A/Caspr colocalizes with Kv1.1 at paranodes during the early stages of myelination. Nogo-A may therefore play a complementary or regulatory role to Caspr in the organization of mature axonal domains and in so doing aid in the co-ordinated localization of K<sup>+</sup> channels to juxtaparanodes (Fig. 8).

Investigation of Nogo-A (or Nogo-A/Caspr) conditional transgenic or knockout animals *in vivo*, possibly using OL specific promoters, may help to further reveal the role of Nogo-A during myelination. According to the inventor's quantitative analyses, the distances of the gap within paired K<sup>+</sup> channel clusters were significantly reduced. This occurs in conjunction with a significant reduction in Nogo-A clusters in both EAE rats and *Shiverer* demyelinated axons compared to normal myelinated axons. These observations imply that K<sup>+</sup> channels co-localize with Caspr again in the pathological conditions, although it would be interesting to explore whether this transient interaction also occurs during remyelination.

A point that also warrants further investigation is the structural and functional nature of the interaction between Nogo-A, Caspr, and the K<sup>+</sup> channels. It should be noted that the localization of Caspr family members demarcate distinct domains in myelinated axons. Caspr2, which is about 45% identical to Caspr, is localized to the juxtaparanodes of adult myelinated

axons. It associates with K<sup>+</sup> channels indirectly via its C-terminus, which contains a putative PDZ binding site (Poliak et al, 1999), a feature shared by two other recently described members of the mammalian Caspr family, Caspr3 and Caspr4 (Spiegel et al, 2002). The C-terminus of Caspr is rather unique compared to other members of the family in terms of its length, and the antibody the inventor has raised is unlikely to cross react with the other Caspr isoforms. The C-terminus of Caspr does not have a putative PDZ binding motif, but shares a band 4.1 binding domain with Caspr2 (Scherer and Arroyo, 2002). It would be interesting to determine if this domain of Caspr mediates its interaction with Kv1.1 and Kv1.2.

#### Nogo-A interactions in the CNS

The only known interacting partners of Nogo-A other than NgR are the Nogo-interacting mitochondrial protein (NIMP) (Hu et al, 2002),  $\alpha$ -tubulin, and MBP (Taketomi et al, 2002). All these interactions are likely to involve the intracellular domains of Nogo-A, and not the Nogo-66 extracellular loop, which is the Nogo-A domain most likely to function as an intercellular signaling ligand. The inventor's results indicate that the oligodendrocyte surface Nogo-66 loop binds directly to axonal surface Caspr, thus implying a previously unsuspected function of Nogo-A at the axoglial junction. This interaction does not appear to involve NgR. In fact, it is unclear if any permanently recurring interaction between adult oligodendroglial Nogo-A and axonal NgR is required under normal physiological conditions. The inventors believe that the Nogo-A/Caspr interaction may in some manner shape and maintain the architecture of the axoglial junctions during and after myelination (Fig.8). More specifically, this interaction may have a role in organizing the location of other molecules at specific junction domains.

In summary, the inventor has shown that oligodendrocyte Nogo-A is clustered at specific axoglial junctions, where it interacts

directly via its extracellular Nogo-66 loop with axonal Caspr,  
and indirectly with K<sup>+</sup> channel proteins. This represents the  
first NgR-independent Nogo-66 interaction described to date, and  
has significant implications for the role of Nogo-A in formation  
5 and maintenance of axoglial junction architecture.

**Materials and Methods Relating to the First Aspect of the  
Invention**

10 **Antibodies**

The polyclonal antibody against Nogo-A was previously described  
(Liu et al, 2002). Two polyclonal antibodies against NgR were  
used in the inventor's experiments. One raised in rabbit with a  
glutathione S-transferase (GST) fusion protein to amino acids  
15 277-430 of human NgR and another a gift from Dr. Stephen  
Strittmatter (Yale University School of Medicine, New Haven; Wang  
et al, 2002). Antibodies against F3 and NB-3 were described  
previously (Ang et al, 2001).

20 To raise polyclonal antibodies against Caspr, a 230bp fragment  
encoding the cytoplasmic region (amino acids 1308-1377) of human  
Caspr (Einheber et al, 1997) was amplified from human brain cDNA  
using primers 5'-AGTCGGATCCACAAAATC ATCGA/CTAT/CA/CAGGG-3'  
(forward) and 5'-ACTCGAATTCAGACCTGGACT CCTCCTCCAA/GGATCTGG-3'  
25 (reverse) with an added *Bam*HI or *Eco*RI site, respectively. The  
amplified fragment was digested with *Bam*HI and *Eco*RI and  
subcloned in-frame into pGEX-3C, and the sequence of the final  
construct was verified by DNA sequencing. The plasmid was  
transformed into *E. coli* BL21, and upon induction a Caspr-GST  
30 fusion protein of the expected size was recovered from bacterial  
lysates using glutathione-agarose beads. Caspr-GST was eluted  
from the beads using reduced glutathione, concentrated by  
lyophilization, and used to immunize rabbits. The immune serum  
obtained from the rabbits was confirmed for its ability to

recognize chick and mouse Caspr through immunoblotting and immunoprecipitation experiments.

5 Mouse monoclonal antibodies against Kv1.1  $\alpha$ -subunit (K20/78) and Na<sup>+</sup> channel (K58/35) were purchased from Upstate Biotechnology and Sigma, respectively. Polyclonal antibodies against Kv1.1, Kv1.2 and Kv2.1 were purchased from Chemicon. The monoclonal anti-MAP2 was purchased from Sigma. The Cy2-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse secondary antibodies  
10 were purchased from Amersham Pharmacia Biotech, and the ABC kit was purchased from Vector Laboratories.

#### EAE Model

15 The Experimental Autoimmune Encephalomyelitis (EAE) model in rats was developed according to a previous report (Ahn et al, 2001). In brief, Lewis rats (2 months old, female) received an injection of 0.5ml/rat of fresh rat spinal cord homogenate (SCH) in complete Freund's Adjuvant (CFA, containing 1mg/ml *Mycobacteria Tuberculosis*; Sigma) (1:1) in the hind footpads bilaterally.  
20 Animals were closely observed for symptoms associated with EAE to determine disease progression. At 13 days post-injection (dpi) ~ 14 dpi, animals at the peak stage of EAE were sacrificed for further experiments.

#### 25 Immunohistochemistry and immunoelectron microscopy

Wistar rats at different postnatal ages (P1, P5, P7, P14, P30, P60 and adults), CGT<sup>-/-</sup> (P16 and P21; Coetzee et al, 1996) and Shiverer (adults; Jackson Laboratories) mice were perfused with 4% paraformaldehyde. The spinal cords and brainstems were removed  
30 and post-fixed in 4% paraformaldehyde for 2 hr, and sequentially incubated in 15% and 30% sucrose. Cryostat sections (10  $\mu$ m) were double-labeled with polyclonal antibodies to Nogo-A (1:200) or Caspr (1:200) in conjunction with a monoclonal Kv1.1  $\alpha$ -subunit (1:200) or Na<sup>+</sup> channel (1:100) antibodies respectively, or with  
35 polyclonal NgR antibodies in conjunction with a monoclonal MAP2



(1:100) or Kv1.1  $\alpha$ -subunit antibodies. Immuno-stainings were visualized and photographed using a Carl Zeiss LSM5 confocal microscope. The lengths for Nogo-A and Kv1.1 immuno-labeling and their overlaps at different postnatal days were measured. Co-localization for Nogo-A and Kv1.1, or Caspr/Kv1.1 at paranodes at different postnatal days was counted. In brainstem sections of normal and EAE rats, wild-type and *shiverer* mice, distances between paired Kv1.1 immuno-labelings were measured and the Nogo-A clusters were counted. Values were presented as mean  $\pm$  SEM. Statistical analyses were carried out using the paired group T-test.

For electron microscopy, samples from adult Wistar rats and CGT<sup>-/-</sup> (P16) were prepared according to published protocols (Huber et al, 2002). Ultrathin sections of 90 nm thickness on nickel grids were blocked for 40 min at room temperature with 1% BSA, 0.1% Tween 20, 1% normal goat serum and 0.025% NaN<sub>3</sub> in 0.1 M sodium phosphate buffer (pH8.3), followed by overnight incubation at 4°C with Nogo-A polyclonal antibodies in the same buffer, respectively. After washing thoroughly with the above buffer, grids were incubated for 1 h with goat anti-rabbit IgG conjugated to 10 nm gold (1:20, Aurion) and fixed with 2.5% aqueous glutaraldehyde for 15 min. After double staining with uranyl acetate and lead citrate, the grids were examined under a Philips 208 electron microscope.

#### Western blot and co-immunoprecipitation assays

From adult, postnatal day 1 (P1) to 30 (P30) Wistar rats, various regions of the CNS (total brain, brainstem, hippocampus, cerebral cortex and spinal cord), spinal cords from EAE and control rats, were harvested and extracted in phosphate-buffered saline containing 1% Triton X-100 and a cocktail of protease inhibitors. Lysates were electrophoresed on SDS-PAGE gel and blotted onto nitrocellulose membranes (Hybond C-extra, Amersham). Identical blots were probed with antibodies against Nogo-A, Caspr, Nogo-66

Receptor (NgR) and  $\gamma$ -tubulin (for loading normalization), and visualized with the Pierce chemiluminescent detection reagents.

For co-immunoprecipitation (IP) experiments, brain membrane  
 5 fractions were prepared as described previously (Lei et al, 2002). Briefly, brain tissue was homogenized in ice-cold homogenizing buffer (320 mM sucrose, 10 mM Tris-HCl pH7.4, 1 mM NaHCO<sub>3</sub> pH7.4, 1 mM MgCl<sub>2</sub>) supplemented with 1% protease inhibitor cocktail (Amersham), and subsequently centrifuged at 5,000 g for  
 10 15 min. The supernatant was collected and spun at 60,000 g (Beckman ultracentrifuge) for 60 min at 4 °C. Pellets were then dissolved in a lysis buffer (10 mM Tris-HCl pH9, 150 mM NaCl, 0.5% Triton X-100, 1% sodium deoxycholate (DOC), 0.5% SDS, 2 mM EDTA, and 1% protease inhibitor cocktail) for subsequent  
 15 experiments. Immunoprecipitated proteins, using of non-immune IgG, Caspr, Nogo-A and NB3 antibodies, respectively, were eluted from the beads with Laemmli sample buffer and separated on 8% SDS-PAGE gels, prior to being transferred to nitrocellulose membrane and probed for Caspr, Nogo-A, Kv1.1, Kv1.2, F3, or NB3.  
 20 In separate experiments, a Nogo-A expression construct in the mammalian expression vector pCIneo (Promega) was transiently transfected into Caspr/F3-expressing CHO (Faivre-Sarrah et al, 2000), F3-expressing CHO (Gennarini et al, 1991) or wild type CHO cells for co-IP studies.

25

#### Cell adhesion assay and PI-PLC treatment

The Nogo-66 peptide (KLSDVLDVLFLLRLEKITCNVHGLASNSYKQVLEES IAVESELYARFPHGEDSKQIAQIVGKYIR) was purchased from Loke  
 30 Diagnostics ApS (Denmark). To generate recombinant proteins of Nogo-66-GST and Nogo-N-terminal-GST (Nogo-N-GST), the encoding sequences for Nogo-66 and Nogo-N- terminus were amplified from human brain cDNA clone HK07722 (Nogo-A) using the primer sets below:

5'-CTGAATTCTTAGGATATACAAGGGTGT-3' (forward)  
 35 5'-GCTAAGCTTTCACTTCAGAGAATCAACTA-3' (reverse) for Nogo-66-GST

5'-AGGAATTCTAGATGAGACCCTTTTGC-3' (forward)

5'-CCCAAGCTTTCAATTAAACTGTCTTTGCTTT-3' (reverse) for Nogo-N-GST.  
The PCR products were digested with *EcoRI* and *HindIII* and ligated into *EcoRI/Hind III*-digested pGEX-KG (Guan and Dixon, 1991).

- 5 Then, these recombinant plasmids were transformed into *E. coli* Top 10 cells. GST fusion proteins were recovered from the bacterial lysates and purified using glutathione-agarose beads. The cell adhesion assay was carried out as previously described (Xiao et al, 1996). Protein spots (1.5 µl of 5 µM GST, Nogo-66-GST  
10 or 100 µM Nogo-66) were applied onto nitrocellulose-coated surfaces of the Petri dishes (Becton Dickinson) and incubated for 2 hours at 37°C in a humidified atmosphere. The dishes were then incubated overnight with PBS containing 2% heat-inactivated fatty acid-free BSA (Sigma) to block residual non-specific protein  
15 binding sites. Mock-transfected CHO, F3-transfected or Caspr/F3 co-transfected CHO cells were then plated in 2 ml of chemically defined medium at a density of  $2.5 \times 10^5$  cells/ml and incubated at 37°C in a humidified atmosphere. After 12 hours, cells were fixed by flooding with PBS containing 2.5% glutaraldehyde. Cells  
20 adhering to the various spots were photographed and counted. All experiments were performed at least three times. Statistical analysis was carried out by Student-t test. The level of significance was chosen at  $p < 0.05$ .

- 25 Where indicated, Caspr/F3-transfected CHO cells were treated with PI-PLC (0.02, 0.04 or 0.06 U/ml) (Sigma), incubated for 2 hours and then plated into the dishes following the same procedures as described above. PI-PLC treated cells were subjected to Western blot analysis and immunochemistry for F3/Contactin as described  
30 above.

#### GST pull-down assay

The bead-bound GST, GST-Nogo-66 and GST-Nogo-N (20 µg) were mixed respectively with 1.5 ml of membrane extracts of adult brain or

of wild type, F3-expressing and Caspr/F3-expressing CHO cells that had been transiently transfected with Kv1.1 (a gift from Dr. Trimmer, Nakahira K et al, 1996) in 0.1 M sodium phosphate buffer (pH7.4) and incubated overnight at 4 °C. The bound proteins were  
5 eluted with 2x Laemmli sample buffer, separated by SDS-PAGE and probed with Caspr and Kv1.1 antibodies.

#### **Lipid rafts analysis**

Preparation of detergent extracts from rat cerebral cortex (15  
10 days and adult) was carried out primarily according to the procedures described by Krämer et al, 1999. In brief, dissected rat cerebral cortex (2g wet weight total) was homogenized in 12.5ml TBS (pH 7.4) containing 2% Triton-X 100, 2mM pervanadate, protease inhibitor tablet (Roche) and stirred for 30 min at 4°C.  
15 The detergent extracts were adjusted to 40% sucrose by adding equal volumes of 80% sucrose in TBS and placed in the ultracentrifuge tube for the SW28 rotor. A linear gradient from 5% to 30% sucrose in TBS were layered onto the lysate sample. Gradients were centrifuged for 18 hrs at 25,000 rpm. Two ml  
20 fractions were collected from top to bottom. Proteins in each fraction were analyzed by SDS-PAGE followed by Western blot. Detergent insoluble floating materials were mostly recovered in fraction 5.

## **25 SECOND ASPECT OF THE INVENTION**

### **Background Information to the Second Aspect of the Invention**

Myelination is a complex multistep process where the underlying molecular mechanism remains far from being completely defined.  
30 However, it is believed that the interaction between axons and myelin competent cells at the paranode play an important role in the insulation of axonal segments in spiral wraps of myelin. This axo-glial contact has been likened to invertebrate septate junctions and has been proposed to serve as an anchor point  
35 between axons and myelin loops, to act as a partial diffusion

barrier into the periaxonal space and to segregate the axon into domains by preventing the lateral diffusion of membrane components (Rosenbluth, 1995). In recent years, specific molecules have been found to be located at the paranodal region.

5

F3/ contactin is a glycosyl-phosphatidylinositol (GPI) linked molecule of the immunoglobulin superfamily of neural cell adhesion molecules (Gennarini *et al*, 1989; Faivre-Sarrailh *et al*, 2000). The molecule is composed of a string of modular  
10 immunoglobulin domains and fibronectin type III repeats. The inventor previously (Xiao *et al*, 1998 incorporated herein by reference) demonstrated that F3 is a neuronal receptor for the extracellular matrix glycoprotein tenascin-R, a glia-derived molecule specifically located at nodes of Ranvier (Wintergerst *et al*, 1993). The binding site on tenascin-R, in its interaction  
15 with F3, was localized to the EGF (epidermal growth factor) like repeats (Xiao *et al*, 1996, 1997, incorporated herein by reference). Tenascin-R is a functional modulator of sodium channel subunits. F3 interacts in *trans* with RPTP $\zeta$ / $\beta$  (receptor  
20 protein tyrosine phosphatase) to promote neurite outgrowth (Sakurai *et al*, 1997) and in *cis* with RPTP $\alpha$  (Zeng *et al*, 1999) to transduce extracellular signals to myelination-related Fyn kinase (Umemori *et al*, 1994). Additionally, F3 co-localizes and  
interacts in *cis* with Caspr/Paranodin and in *trans* with glial  
25 neurofascin 155 at the paranode (Girault and Peles, 2002), a key site of axoglial contact for myelination. F3 null mice exhibit partially disrupted paranodal structure and die by P18 (Berglund *et al*, 1999), suggesting that F3 may be critical for development.  
30 F3-associated protein (Caspr), also known as paranodin, is an additional axonal component of the paranode (Einheber *et al*, 1997; Menegoz *et al*, 1997). Rios *et al* (2000) further showed that F3 and Caspr co-localize and interact in a *cis* fashion at the paranode during myelination. In adult sciatic and optic nerves,

F3 staining localized to the paranodes, although staining also extended to the nodes in the optic nerve.

NB-3 is a neural cell adhesion molecule in the same subfamily as F3 (Lee *et al*, 2000). In the cerebrum, NB-3 mRNA analysis revealed a low level of expression during embryogenesis with an abrupt increase in the postnatal period, reaching a maximum level in the postnatal seventh day which corresponds to the time frame for myelination. Subsequently, levels decreased to one-fifth of the peak and remained so in adulthood.

Myelination in the vertebrate central nervous system (CNS) is essential for rapid impulse conduction. In the CNS, oligodendrocyte (OL) differentiation is mediated by neuron derived signals (Barres and Raff, 1999). Jagged1/Notch1, an axoglial interaction, promotes oligodendrocytes precursor cell (OPC) migration and inhibits OPC differentiation (Wang *et al*, 1998). Notch/Jagged1 signaling pathway plays a critical role in promoting gliogenesis, such as radial glial cells in the fetal forebrain, Schwann cells in dorsal root ganglia, and Müller glial cells in the retina (Furukawa *et al*, 2000; Hojo *et al*, 2000; Gaiano *et al*, 2000; Morrison *et al*, 2000; Wakamatsu *et al*, 2000; Tanigaki *et al*, 2001). Conditional ablation of Notch1 in OPCs results in the appearance of ectopic premature OLs and subsequent apoptosis (Genoud *et al*, 2002) and the failure of efficient remyelination is partly attributed to the activation of OPC Notch receptor by astrocyte-expressed Jagged1 in multiple sclerosis (John *et al*, 2002) indicating that OPC differentiation could be disordered when Notch1 is absent or inadequately activated by Jagged1. Thus, other pathways via Notch1, besides the inhibitory Jagged1/Notch1 signalling pathway, may instructively mediate OPC differentiation into OLs. However, the molecular mechanisms controlling the timing of OPC differentiation to OLs and the subsequent OL maturation remain poorly defined. Given that Jagged1 is downregulated significantly before the maturation of

oligodendrocytes, it is conceivable that other molecules may interact with Notch, which continually plays a role in myelination. Molecules congregated at distinct segments of the axon are potential Notch1 ligands.

5

Notch is a type I transmembrane protein mediating cell fate selection via lateral inhibition. Its core signaling mechanism involves Regulated Intramembrane Proteolysis (RIP) (Ebinu and Yankner, 2002). Upon binding the classic ligands, Delta,  
10 Serrate/Jagged and Lag-2 (collectively called DSL), Notch undergoes two proteolytic cleavages that release its intracellular domain (NICD). NICD translocates to the nucleus and interacts with RBP-J transcription factor to activate, for instance, *Hes* genes (Martinez Arias et al, 2002). In addition,  
15 *Deltex1* (DTX1) has been identified as a cytoplasmic downstream element of the Notch signaling pathway. DTX homologs share three common domains, namely, the N-terminal region, proline-rich and RING-H2 finger motifs (Kishi et al, 2001). Particularly, the N-terminal region interacts with NICD. Notch signaling via DTX1  
20 represses JNK signaling, a pathway regulating OL differentiation, and cooperates with Wingless signaling (Brennan and Gardner, 2002; Martinez Arias et al, 2002). Although several studies imply the existence of an extracellular ligand that activates Notch/DTX1 signaling, the putative ligand has not yet been  
25 identified.

Notch has been shown to regulate glial differentiation (Wang et al, 1998; Gaiano et al, 2000; Morrison et al, 2000). Of significance to the inventor was that the Notch extracellular  
30 portion contains, as does tenascin-R, multiple EGF-like repeats which are sites of potential ligand-receptor interactions (Rebay et al, 1991). It thus becomes conceivable to the inventor that glia-derived Notch could be a binding partner of axonal F3 and NB-3. In the rat optic nerve, Wang et al (1998) demonstrate  
35 *Jagged1* expression on retinal ganglion cell axons. *Jagged1*

signals to Notch on oligodendrocyte precursors to inhibit their differentiation. Of interest is the expression pattern of Jagged1 which becomes developmentally downregulated with a time course that parallels myelination (Dugas et al, 2001). This led to the conclusion that Jagged1 signals to oligodendrocytes thus as part of a localized timing mechanism to regulate oligodendrocyte differentiation and thus myelination. But how is the segmental nature of the myelin sheath preserved? What is the stop signal that prevents myelinating oligodendrocytes from encroaching upon putative nodes of Ranvier? The inventor hypothesized that such axonal stop signals should logically exist on either side of the nodes, namely at the paranodes. As detailed below, the inventor has indeed demonstrated that F3 is able to act as a stop signal for oligodendrocyte processes and that NB-3 may participate in the triggering of oligodendrocyte differentiation.

#### Summary of the Second Aspect of the Invention

The present inventor has for the first time shown that both F3 and NB-3 are physiological ligands of the oligodendroglial Notch receptor - establishing the presence of a signalling pathway via Deltex1 to co-ordinate events during myelination.

Thus, the inventor has identified a new paranodal molecule - NB-3 and showed that stop signals are located at paranodes which involve F3/NB-3 signalling to Notch on the surface of oligodendroglia. In a co-culture system between OLN-93 cells and F3-transfected CHO cells, oligodendroglial cellular processes terminate and spread over the F3-transfected cell bodies but bypass control CHO cells. Cell adhesion, co-immunoprecipitation and GST pull-down assays confirm that F3/NB-3 and Notch associate as a complex. MAG becomes upregulated when OLN-93 cells and F3-transfected CHO cells are co-cultured and when OLN-93 cells and primary oligodendrocytes contact F3 and NB-3 protein substrates. These results describe a novel and functionally significant signalling interaction between F3/NB-3 and Notch that is involved



in the regulation of myelination. The present inventor also shows in cell adhesion tests and biochemical assays that F3 is able to bind to Notch1 and Notch2. Furthermore the interaction induces radical morphological change in oligodendrocyte cell line OLN-93  
5 which develops the ensheathing feature and significantly upregulates myelin-related proteins, such as MAG (myelin-associated glycoprotein), CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase), and PLP. These results suggest that F3 is a physiological ligand of Notch receptor and the signaling plays an  
10 important role in oligodendrocyte maturation.

This determination shows that F3 and NB-3 interaction with Notch plays an important role in oligodendrocyte maturation.

15 The inventor has further determined that F3 induces Notch intramembrane cleavage at the S3 site and that F3/Notch-induced MAG upregulation is independent of HES1 and involves Deltex 1 (DTX1). DTX1 is a known cytoplasmic downstream element of the Notch signaling pathway. It has previously been shown that Notch  
20 signaling via DTX1 represses JNK signalling, a pathway that regulates oligodendrocyte differentiation. However, the previous studies have not identified the extracellular ligand that activates Notch/DTX1 signaling.

25 Here the inventor reports that NB-3 is a neuron-derived cell recognition molecule developmentally clustering at the CNS paranodes and identify NB-3 as a functional ligand of Notch1. The NB-3/Notch signalling pathway promotes OPC differentiation and OL maturation via Deltex1. Moreover, Jagged1 is localized to  
30 juxtaparanodes and internodes. Thus a spatial signal switch mechanism from Jagged1/Notch1 to NB-3/Notch1 may exist along the axon, which coordinates oligodendroglial maturation.

Neurons and glia in the vertebrate CNS arise in temporally  
35 distinct, albeit overlapping phases. Neurons are generated first,

followed by astrocytes and then oligodendrocytes (OLs) from common progenitor cells (Sauvageot and Stile, 2002). Increasing evidence indicates that axon-derived signals are temporally and spatially required for modulating OL maturation as well as myelin formation (Barres and Raff, 1999; Hu et al, 2003). However, little is known about how neuronal molecules participate in OL generation from neural stem cells (NSCs). The inventor's investigation on how to favorably direct embryonic neural stem cells to differentiate into OLs showed that NB-3, a neuronal cell recognition molecule, bound to Notch1 and triggered nuclear translocation of the Notch1 intracellular domain in the stem cells. This NB-3/Notch1 interaction promotes oligodendroglialogenesis from embryonic neural stem cells, which can be blocked by dominant-negative Notch1 and deletion mutants of Deltex1 that lacks the Ring-H2 motif. However, constitutively active Notch1 alone fails to promote OL generation, suggesting that NB-3-induced NICD is required in this event. Taken together, the observations here demonstrate that the NB-3/Notch1 signalling pathway via Deltex1 instructs oligodendroglialogenesis.

20

Thus the present invention provides a method of stimulating differentiation of an oligodendrocyte or precursor thereof, comprising contacting said oligodendrocyte or precursor with F3, NB-3, or a mimetic of either.

25

The present invention further provides a method of stimulating myelination of a neuron, specifically a neural axon, comprising contacting an oligodendrocyte, a precursor thereof, or a neuron, with F3, NB-3, or a mimetic of either.

30

In either of the methods described above, the oligodendrocyte, precursor, or neuron as appropriate, is preferably contacted with both F3 and NB-3, or mimetics thereof. F3 and NB-3 may be present as a complex.

Expression of myelin proteins, such as MAG, will typically be upregulated in said oligodendrocyte or precursor in response to the contacting step.

5

Without wishing to be bound by any particular theory, it is believed that upregulation of MAG is induced through binding of F3, NB-3 or mimetics thereof to Notch, particularly to Notch 1 or 2, on the surface of the oligodendrocyte or precursor thereof.

10 Such binding is believed to induce Notch signalling, via Deltex-1.

The oligodendrocyte precursor may be an oligodendroglial precursor cell (OPC) or a neural stem cell (NSC). OPCs typically display CNPase, Gal C and MAG on their surface; NSCs display Nestin marker. OPCs and NSCs are described by Wang et al, 1998 (Notch receptor activation inhibits oligodendrocyte differentiation. Neuron 21, 63-75); Morrison S.J., 2001 (Neuronal potential and lineage determination by neural stem cells. Curr. Opin. Cell Biol. 13, 666-672); Sauvageot, C.M. and Stiles, C.D., 2002 (Molecular mechanism controlling cortical gliogenesis. Curr. Opin. Neurobiol. 12, 244-249); Xiao et al, 2003 (F3/Contactin is a notch ligand. Cell, Vol 115, 163-175).

25 The methods may be performed in vitro or ex vivo. The methods are particularly applicable to the generation of differentiated oligodendrocytes which may be used for therapeutic purposes. Thus, after said contacting step, the OPC or NSC may be introduced or implanted into a subject having disease of, or injury to, the central nervous system; for example, any condition characterised by demyelination of neurons, such as multiple sclerosis (MS), epilepsy, stroke and spinal cord injury (SCI). Following introduction or implantation, these treated cells may continue to differentiate and provide myelination for de-

35 myelinated neurons.

It may be desirable to obtain the oligodendrocyte precursor cells from the subject to whom they are to be administered after treatment.

5

The invention further provides a composition comprising F3 and NB-3, or mimetics thereof, in combination with a carrier. The composition may comprise a complex between F3 and NB-3, or a mimetic thereof.

10

In certain embodiments, the composition is a pharmaceutical composition, and so comprises a pharmaceutically acceptable carrier. Preferably pharmaceutical compositions are formulated for injection in vivo, and still more preferably for direct injection into the CNS.

15

Also provided are compositions as described above for use in a method of medical treatment. In particular the compositions are provided for use in the treatment of injury to, or disease of, the CNS. They may be used for treatment of any condition characterised by demyelination of neurons, such as SCI, MS, epilepsy or stroke.

20

Also provided is the use of F3 and/or NB-3 in the preparation of a medicament for the treatment of injury or disease to the CNS. They may be formulated individually or separately. Separately formulated NB-3 and F3 may nevertheless be administered together.

25

Thus there is also provided the use of F3 in the preparation of a medicament for the treatment of injury or disease to the CNS, wherein the medicament is for administration in combination with NB-3 or a mimetic thereof.

30

Likewise, there is provided the use of NB-3 in the preparation of a medicament for the treatment of injury or disease to the CNS,

35

wherein the medicament is for administration in combination with F3 or a mimetic thereof.

5 The invention further provides a method of stimulating myelination of a neuron, specifically a neural axon, comprising contacting a neuron or an oligodendroglial cell with a composition as described herein.

10 The invention further provides a method of treating a subject having disease of, or injury to, the central nervous system, comprising administering to the subject a pharmaceutical composition as described herein.

15 As will be clear from the foregoing, the subject will typically have a condition characterised by demyelination, such as SCI, MS, epilepsy or stroke.

20 The invention further provides a method of screening for a substance capable of modulating (preferably promoting) interaction between Notch and F3 and/or NB-3, the method comprising contacting F3 and/or NB-3, Notch and a candidate substance, and determining the interaction between Notch and F3 and/or NB-3.

25 The method may further comprise contacting Notch and F3 and/or NB-3 in the absence of the candidate substance under otherwise analogous conditions, and determining the interaction between Notch and F3 and/or NB-3.

30 Preferably the method comprises contacting a complex between Notch and F3 and/or NB-3 with the candidate substance. The complex is preferably formed before it is contacted with the candidate substance.

The method may be performed by any appropriate method. The skilled person will be well aware of many suitable assay formats, and will be capable of designing further examples.

- 5 Any or all of NB-3, F3 and Notch may be present in, or on, a cell. The gene from which the protein is expressed may be endogenous to the cell in question, or it may be present on a vector introduced into the cell. The protein is preferably expressed on the surface of the cell.
- 10 Additionally or alternatively, any or all of NB-3, F3 and Notch may be immobilised on a solid support. One or both may comprise a detectable label as described in more detail below.
- 15 In particular embodiments, Notch is present on a cell surface, and the method comprises determining Notch signalling, particularly via Deltex 1. If the cell in question is an oligodendrocyte or precursor thereof, the method may comprise determination of upregulation of MAG expression.
- 20 The invention further provides a method of manufacturing a pharmaceutical formulation comprising, having identified a substance capable of modulating interaction between Notch and F3 and/or NB-3 by a screening method described herein, the further
- 25 step of formulating said substance with a pharmaceutically acceptable carrier. The method may comprise the further step of optimising said identified substance for administration in vivo prior to formulation.
- 30 In specific embodiments, the present invention firstly provides a method for enhancing myelination in an individual comprising administering to said patient an activating agent of Notch receptor, said activating agent comprising F3, NB-3, or a mimetic thereof.
- 35

The method is preferably used in the treatment of SCI, MS, epilepsy or stroke.

The invention also provides a pharmaceutical composition  
5 comprising an activating agent of a Notch receptor, said activating agent comprising F3, NB-3, or a mimetic thereof.

Also provided is a method of screening for substances capable of modulating the interaction between a ligand and a Notch receptor,  
10 the ligand being F3, NB-3, or a mimetic thereof, comprising contacting a substance with the ligand and the receptor; determining the interaction between the ligand and the receptor and comparing this with the interaction between the receptor and ligand under comparable conditions but in the absence of said  
15 substance.

The method may further comprise producing a pharmaceutical composition containing said substance.

## 20 Cells

The term oligodendrocyte is used herein to refer to oligodendroglial cells capable of laying down a myelin sheath around a neuronal axon in the central nervous system (CNS).  
25

The term oligodendrocyte precursor cell is used to refer to cells capable of differentiating into oligodendrocytes on administration of suitable stimuli, such as F3 and NB-3. Such cells include oligodendroglial precursor cells (OPC) and neural  
30 stem cells (NSC).

## Protein sequences

The term "Notch" is used to encompass all isoforms of the Notch  
35 protein, including Notch 1, 2 and 3, as well as portions and

isolated domains thereof, such as the extracellular domain, as well as mutants and variants thereof having greater than 80%, 85%, 90%, 95%, 96%, 97% 98% or 99 % identity to the sequence given below. Preferred proteins are Notch 1 and 2. Orthologous proteins from other mammalian species are also included. Preferably the Notch protein has the ability to bind to a NB-3 and/or a F3 protein. It may also have the ability to induce upregulation of MAG protein expression via Deltex 1 signalling in an oligodendrocyte or precursor thereof.

The term "F3" is used to encompass isoforms of the F3 protein, and isolated domains of such proteins, as well as mutants and variants thereof having greater than 80%, 85%, 90%, 95%, 96%, 97% 98% or 99 % identity to the sequence given below. Orthologous proteins from other mammalian species are also included. The F3 protein preferably has the ability to bind to the extracellular domain of a Notch protein, particularly of Notch 1 and 2.

The term "NB-3" is used to encompass isoforms of the NB-3 protein, and isolated domains of such proteins, as well as mutants and variants thereof having greater than 80%, 85%, 90%, 95%, 96%, 97% 98% or 99 % identity to the sequence given below. Orthologous proteins from other mammalian species are also included. The NB-3 protein preferably has the ability to bind to the extracellular domain of a Notch protein, particularly of Notch 1 and 2.

The amino acid sequences of Notch, F3 and NB-3 proteins are shown below, along with their GenBank accession numbers;

F3/Contactin

gi:414791 (CAA79696)

```

1  mkmwllvshl viisittcla eftwyrrygh gvseedkgfg pifeeqpint iypeeslegk
61  vslncraras pfpvykrmn ngdvdltedr ysmvggnlvi nnpdkqkdag iyyclasnny
121 gmvrsteatl sfgyldpfpp eerpevrveke gkgmvllcdp pyhfpddlsy rwllnefpvf
181 itmdkrrfvs qtngnlyian veasdkgnys cfvsspsitk svfskfipli piperttkpy
241 padivvqfkd vyalmgqnv tlecfa lgnpvp pdirwrkvle pmpstaeist sgavlkifni

```



301 qledegiyec eaenirgkdk hqariyvqaf pewvehindt evdigsdlyw pcvatgkpi  
 361 tirwlkngya yhkgeirlyd vtfenagmyq ciaentygai yanaelkila laptfemnpm  
 421 kkkilaakgg rviieckpka apkpkfswsk gtewlvnssr iliwedgsle innitrndgg  
 481 iytcfannenr gkanstgtlv itdptriila pinaditvge natmqcaasf dpaldltfvw  
 5 541 sfngyvidfn kenihyqrf mldsnigelli rnaqlkhagr ytctaqtivd nssasadlvv  
 601 rgppgppggl riediratsv altwsrgsdn hspiskytiq tktilsddwk daktdppie  
 661 gnmeaaravd lipwmeeyfr vvatntlgrg epsipsnrik tdgaapnvap sdvgggggrn  
 721 reltitwapl sreyhygnnf gyivafkpfid geewkkvtvt npdtgryvhk detmspstaf  
 781 qvkvkafnnk gdgpysllav insaqdapse aptevgvkvl ssseisvhwe hvlekiyesy  
 10 841 qirywaahdk eeeanrvqvt sgeysarlen llpdtqyfie vgacnsagcg ppsdmieaft  
 901 kkappsqppr iissvrsgsr yiitwdhvva lsnestvtgy kvlyrpdgqh dgklysthkh  
 961 sieviprdg eyvvevrahs dggdgvvsqv kisgaptlsp slglillpaf gilvylef

15

NB3 gi:5631291 (BAA82612)

1 mrlwlklvil lplinsagdg gllsrpiftq ephdvifpld lsksevilnc aangypsphy  
 61 rwkqngtdid ftmsyhyrld ggslainsph tdqdigmyqc latnllgtl srkaklqfay  
 20 121 iedfekttrs tvsvregqgv vllcgppphf gdlsyawtfn dnplyvqedn rrfvsqetgn  
 181 lyiakvepsd vgnycfitn keaqrsvqgp ptplvqrtgd vmgeyepkie vrfpetiqaa  
 241 kdssvklecf algnvpvdis wrldgspplp gkvkysksqa ileipnfqqe degfyecias  
 301 nlrgrnlakg qlifyappew eqkiqnthls iydnlleweck asgkpnpyt wlkngrlnp  
 361 eeriqiengt liitmlnvds sgvyqcaeen kyqiiyanae lrvlasapdf skspvkkksf  
 25 421 vqvvgdivig ckpnafpraa iswkrgetl rskriflle dgsikiynit rsdagsytci  
 481 atnqfgtakn tgslivkert vitvppskmd vtvgesivlp cqvsdpsie vfvwffngd  
 541 vidlkkgvah feriggesvg dlmirniqlh hsgkylctvq ttleslsava diivrgppgp  
 601 pedvqvedis sttsqlswra gpdnnspiqi ftiqtrtpfs vgwqavatvp eilngktyna  
 661 tvvgldspwve yefrvvagns igigepseps ellrtkasvp vvpvniagg ggsrselvit  
 30 721 wesipeelqn gegfyiimf rpvgsttwsd ekvssvessr fvyrnesiip lspfevkvgv  
 781 ynnegegsls tvtivysged epqlaprgts lqsfaseme vswnaiawnr ntgrvlgyev  
 841 lywtddskes migkirvsng vttnitgk antiyfasvr ayntagtgps sppvnvttkk  
 901 sppsqppani awkltnsklc lnwehvtme nesevlgyki lyrqnrqskt hiletntsa  
 961 ellvpfeedy lieirtvsdg gdgssseier ipkmsslssr giqflepst flsivivifh  
 35 1021 cfaiqlpi

Notch1 gi:11275980 (AAG33848)

40 1 mppllapllc lallpalaar gprcsqpget clnggkceaa ngteacvcgg afvgprcqp  
 61 npclstpcn agtchvdrx gvadyacsa lgfsgplclt pldnacitnp crnggtcdll  
 121 tlteykrp pgwsgkscq adpcasnpc nggqclpfea syichcppsf hgptcrqdv  
 181 ecgqkprlcr hggtnhnevg syrcvcrath tgpncerpyv pcspspcng gtrcptgdvt  
 241 hecacplpft gqnceenidd cpnncnngg acvdgvntyn cpcppewtgq yctedvdecq  
 45 301 lmpnacqngg tchnthggyn cvcvgwtge dcseniddca saacfhgac hdrvasfyce  
 361 cphgrtgllc hlndacisnp cnegsnctdn pvngkaictc psqytpacs qdvdecslga  
 421 npcehagkci ntigsfecqc lqgytgprce idvnecvsnp cqndatcldq igefqcmcmp  
 481 gyegvhcevn tdecasspcl hngrcldkin efqecptgf tghlcqydv ecastpckng  
 541 akcldgpnty tcvctegygt thcevdidec dpdpchygsc kgdgvatftcl crpgytghhc  
 50 601 etninecssq pcrirgtcqd pdnaylcfcl kgttgpncei nlddcasspc dsqclclkid  
 661 gyeceapgy tgsmcnsnid ecagnpchn gtcceingf tcrcepyghd ptclsevnec  
 721 nsnpcvhgac rdslnykdcd cdpwsgtnc dlnnecesn pcvnggtckd mtsgivctcr  
 781 egfsgpncqt ninecasnnc lnkgctiddv agykcncllp ytgatcevv apcspcprn  
 841 ggecrqsedy esfscvcpa gakgtcevd inecvlspcr hgascqnthg xyrchcagy  
 55 901 sgrncetdid dcrpnpchn gscldginta fcdclpgfrg tfceedinec asdpcran  
 961 ctdecdsytc tcapagfsgih cenntpdcte sscfnggtcv dginsftclc ppgftgscq  
 1021 hvvnecdsrp cllggtcqd rglhrctcpq gytgpncln vhwcdsspck nggkewqtht

1081 qyrcecpqsgw tglycdvpsv scevaagrqq vdvarlcqhg glcvdagnth hrcrcqagytg  
 1141 sycedlvdec spspcngat ctdylggysc kcvagyhgvn cseeidecls hpcqnggtcl  
 1201 dlpntykcsc prgtqgvhce invddcnppv dpvsrspkcf nngtcvdvqg gysctcpggf  
 1261 vgercegdvn eclsnpcdar gtqncvqrwn dfhcecragh tgrrcesvin gckgkpcng  
 5 1321 gtcavasnta rgfickcpag fegatcenda rtcgsrlrcn ggctcisgprs ptclclgpf  
 1381 gpecqfpass pclggnpcyn qgtceptses pfyrclcpak fngllchild ysfgggagr  
 1441 ippplieeac elpecqedag nkvcslqcn hacgwdggdc slnfndpwkn ctqslqcwky  
 1501 fsdghcdsqc nsagclfdgf dcqraegqcn plydqyckdh fsdghcdqgc nsaecewdgl  
 1561 dcaehvperl aagtlvvvvl mppeqlrns fhflrelsr lhtnvfkrd ahgqgmifpy  
 10 1621 ygreelrkh pikraaegwa apdallgqv asllpggseg grrrrreldpm dvrgsivyle  
 1681 idnrqcvqas sqcfqsatdv aafllgalasl gslnipykie avqsetvepp ppaqlhfmv  
 1741 aaaaflvllff vgcgvllsrk rrrqhglwlf pegfkvseas kkkrrreplge dsvglkplkn  
 1801 asdgalmddn qnewgdedle tkkfrfeepv vlpdllddgt hrgwtqghld aadlrmsama  
 1861 ptpqggevda dcmdvnrvgp dgftplmias csaggletgn seeedapav isdfiyqgas  
 15 1921 lhnqtdrtge talhlaarys rsdaakrlle asadaniqdn mgrtphlaav sadaqgvfqi  
 1981 linratdld armhdgttpl ilaarlaveg mledlinsha dvnvddlgk salhwaav  
 2041 nvdaavvllk ngankdmqnn reetplflaa regsyetakv lldhfanrd tdhmdrlpr  
 2101 iaqermhhdv vrlldeynlv rspqlhgapl ggtptlsppl cspngylgsi kpgvqgkvr  
 2161 kpskglacg skeakdlkar rkksqdgkyc lldssgmisp vdslesphgy lsdvasppl  
 20 2221 pspfqspsv plnhlpgmpd thlgighlnv aakpemaalg gggrlafetg pprrlshlpva  
 2281 sgtstvlgs sggalnftvg gstslnqgce wlsrlqsgmv pnqynplrgs vappglstqa  
 2341 pslqhgmvpg lhsslaasal gsmmsyqgip strlatqphl vqtqgvqpqn lqmqqqnlqp  
 2401 aniqqqqslq ppppppphl gvssaasghl grsflsgeps qadvqplgps slavhtilpq  
 2461 espalptslp sslvppvtaa qfltpspqhs ysspvdntps hqlqvpehpf ltpspespdq  
 25 2521 wssssphsnv sdwsegvssp ptsmqsqiar ipeafk

Notch2

gi:11275978 (AAA36377)

30 1 mpalrpallw allalwlcca apahalqcrd gyepcvnegm cvtyhngtgy ckcpgefllge  
 61 ycqhrrdpcek nrcqnggtcv aqamlgkatc rcasgftged cqystshpcf vsrplnggt  
 121 chmlsrdtye ctcqvfgtgk ecqwtadcls hpcangstct tvanqfscck ltgftgqkce  
 181 tdvnecdipg hcqhgggtcln lpgsyqcqcp qgftggyqcds lyvpcaspc vnggtcrqtg  
 241 dftfecncpl gfegstcern iddcphrcq nggvcvdgvn tyncrcppqw tgqfctedvd  
 35 301 ecllqpnaq nggtcanrng gygcvcvngw sgddcsenid dcafasctpg stcidrvasf  
 361 scmcepgkag llchlddaci snpchkgalc dtnplnggyi ctcpggykga dtedvdeca  
 421 mansnpceha gkcvntdgaf hceclkgayv prcemdinec hsdpcqndat cldkiggftc  
 481 lcmpgfkghv celeinecqs npcvnnqgcv dkvnrfqclc ppgftgpvcq ididdcsstp  
 541 clngakcidh pngyecqcat gftgvlceen idncdpdpch hgqcqdgids ytcicnpgym  
 40 601 gaicsdqide cysspcldng rclidlvngyq cncqpgtsgv nceinfddca snpcihgicm  
 661 dginyrscvc spgftgqrn ididecasnp crkgatcing vngfricpe gphhpscysq  
 721 vneclsnpci hgntgglsy ykclcdagwv gincevdkne clsnpcqngg tcdnlvngyr  
 781 ctckkgfkgy ncqvnideca snpcnqgtc fddisgytch cvlpytgknc qvlapcspn  
 841 pcenaavcke spnfesytcl capgwqgqrc tididecisk pcmnhglchn tqgsymcecp  
 45 901 pgfsgmdcee diddclanpc qnggscmdgv ntfscclclpg ftgdkcqtmd neclsepckn  
 961 ggtcsdyvns ytckcagfd gvhcennine ctesscfngg tcvdginsfs clcpvgftgs  
 1021 fclheinecs shpcnlegtc vdglgtyrcs cplgytgknc qtlvnlcsrs pcknkgctcv  
 1081 kkaesqclcp sgwagaycdv pnvsdiaas rrgvlvehlc qhsgvcinag nthycqcp  
 1141 ytgsyceeql decasnpqg gatcsdfigg yrcecvpgyq gvnceyevde cqnpqpcqngg  
 50 1201 tcidlvnhfk cscppgtrgl lceeniddca rgphclnggq cmdriggysc rclpgfager  
 1261 cegdinecls npcsegsld ciqltndylc vcrsaftgrh cetfvdvcpq mpclnggtca  
 1321 vasmnpdgfi crcppgfsa rcqsscqvht crkgeqcvht asgprcfvps prdcesgcas  
 1381 spcqhggsc pqrpppyysc qcappfsgsr celytappst ppatclsqyc adkardgvcd  
 1441 eacnshacqw dggdcaltme npwancsspl pcwdyinnqc delontvecl fdnfecqgns  
 55 1501 ktckdykca dhfkdnhcnc gcnscecgwd gldcaadqpe nlaegtllviv vlmppqqlq  
 1561 darsflralg tllhtnlrik rdsqgelmv pyygeksaam kkqrmtrrsi pgeqeqevag  
 1621 skvfleidnr qcvqsdhcf kntdaaaall ashaiggtls yplsvsvses ltpertqilly

1681 llavavviil fiillgvima krkrkhgslw lpegftlrrd asnhkrrepv gqdavglknl  
 1741 svqvseanli gtgtsehvwv deggpqpkvk aedeallsee ddpidrrpwt qqhleaadir  
 1801 rtpslaltpp qaeqevdvld vnvrgpdgct plmlaslrvg ssdlssdeded aedssaniit  
 1861 dlvyqgaslq aqtdrtgema lhlaarysra daakrlldag adanaqdnmg rcplhaavaa  
 5 1921 daqgvfqiili rnrvtlddar mndgttliil aarlavegm v aelincqadv navddhgksa  
 1981 lhwaavnnv eatllllkng anrdmqdnke etplflaare gsyeaakill dhfanrditd  
 2041 hmdrlprdva rdmhhddivr lldeynvtps ppgtvltsal spvicgpnrs flslkhtpmg  
 2101 kksrpsaks tmptslpnla keakdakgsr rkkslsekvg lsessvtlsp vdslesphty  
 2161 vsdttsspmi tspgilqasp npmlataapp apvhaqhals fsnlhemqpl ahgastvlp  
 10 2221 vsqllshhhi vspqsgsags lsrlhvpvpv adwmnrmevn etqynemfgm vlapaegthp  
 2281 giapqsrppe gkhittprep lppivtfqli pkgsiaqpag apqpqstcpp avagplptmy  
 2341 qipemarlp vafptammpq qdgqvaqtil payhpfpsv gkytppsgh syassnaaer  
 2401 tpsghlqg ehpyltpspe spdqwsssp hsasdwsvt tsptpggagg gqrgpgthms  
 2461 epphnmqvy a

### Assay methods

As described above, the skilled person is well aware of numerous  
 20 assay formats which may be appropriate for determining  
 interaction between Notch and NB-3 and/or F3, and identifying  
 substances which modulate, preferably promote, such interaction.

For example, interaction between the proteins may be studied in  
 25 vitro by labelling one or more with a detectable label and  
 bringing it into contact with another which has been immobilised  
 on a solid support. Suitable detectable labels, especially for  
 petidyl substances include <sup>35</sup>S-methionine which may be  
 incorporated into recombinantly produced peptides and  
 30 polypeptides. Alternatively the complex formed on the solid  
 support may be detected by labelling with an antibody directed  
 against an epitope present on a protein which is not immobilised  
 on the solid support. If no suitable antibody is available, a  
 recombinantly-produced peptide or polypeptide may be expressed as  
 35 a fusion protein containing an epitope against which a suitable  
 antibody is available.

The protein which is immobilized on a solid support may be  
 immobilized using an antibody against that protein bound to a  
 40 solid support or via other technologies which are known per se.

A preferred in vitro interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an in vitro assay format of the type described above a test compound can be assayed  
5 by determining its ability to affect the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to  
10 remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

An assay according to the present invention may also take the  
15 form of a cell-based assay in which at least one of the proteins is expressed by, preferably on the surface of, a suitable cell. The assay may utilise a cell line, such as a yeast strain or mammalian cell line, in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into  
20 the cell.

Modulators of Notch/NB-3/F3 interaction identified by the methods described may be further modified to increase their suitability for in vivo administration.

25

#### Formulations

The compositions of the invention may be prepared as pharmaceutical formulations comprising at least one active  
30 compound, as defined above, together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, including, but not limited to, pharmaceutically acceptable carriers, adjuvants, excipients, buffers, preservatives and stabilisers. The formulation may  
35 further comprise other active agents.

Thus, the present invention further provides a method of making a pharmaceutical composition as previously defined, the method comprising admixing at least one active agent as described herein together with one or more pharmaceutically acceptable ingredients well known to those skilled in the art, e.g., carriers, adjuvants, excipients, etc..

The term "pharmaceutically acceptable" as used herein pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of the subject in question (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, adjuvant, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

Formulations may suitably be injectable formulations, e.g. in the form of aqueous, isotonic, pyrogen-free, sterile solutions, in which the active compound is dissolved. Such liquids may additionally contain other pharmaceutically acceptable ingredients, such as anti-oxidants, buffers, preservatives, stabilisers, bacteriostats, suspending agents, thickening agents, and solutes which render the formulation isotonic with the blood or cerebrospinal fluid. Examples of suitable isotonic carriers for use in such formulations include Sodium Chloride Injection, Ringer's Solution, or Lactated Ringer's Injection. Typically, the concentration of the active compound in the liquid is from

about 1 ng/ml to about 10 µg/ml, for example from about 10ng/ml to about 1µg/ml. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition  
5 requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.

10 Administration

Administration of the compositions of the invention will generally be by injection, preferably directly into the CNS. Injection may be directly into the site of damage.  
15 Alternatively, injection may be into the cerebro-spinal fluid, typically near the site of injury or illness.

Sequence identity

20 Percent (%) amino acid sequence identity with respect to a reference sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the  
25 maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. % identity values may be determined by WU-BLAST-2 (Altschul et al., Methods in Enzymology, 266:460-480 (1996)). WU-BLAST-2 uses several search parameters, most of which are set to the default  
30 values. The adjustable parameters are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11. A % amino acid sequence identity value is determined by the number of matching identical residues as determined by WU-BLAST-2, divided by the total number of residues

of the reference sequence (gaps introduced by WU-BLAST-2 into the reference sequence to maximize the alignment score being ignored), multiplied by 100.

- 5 Percent (%) amino acid similarity is defined in the same way as identity, with the exception that residues scoring a positive value in the BLOSUM62 matrix are counted. Thus, residues which are non-identical but which have similar properties (e.g. as a result of conservative substitutions) are also counted.

10

- In a similar manner, percent (%) nucleic acid sequence identity with respect to a reference nucleic acid is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the reference  
15 nucleic acid sequence. The identity values used herein may be generated by the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

20 **The subject**

- The subject to which the compositions and/or treatments of the invention will be administered will be a mammal, preferably an experimental animal such as a rodent (e.g. a rabbit, rat or  
25 mouse), dog, cat, monkey or ape, or a farm animal such as a cow, horse, sheep, pig or goat. More preferably, the subject is human.

- Generally, the subject will have CNS damage, usually resulting  
30 from a disease or disorder characterised by inadequate myelination. Such conditions include MS. In experimental animals, the damage or disorder may be experimental. The CNS damage may also result from physical injury e.g. spinal cord injury (SCI) other diseases or disorders, e.g. stroke, epilepsy

or a neurodegenerative condition, learning memory-related condition and/or dementia such as Alzheimer's disease or Parkinson's disease.

- 5 The treatments of the invention may be used in conjunction with other therapies, such as surgery and/or rehabilitation.

#### Mimetics

- 10 Non-peptide "small molecules" are often preferred to peptides or polypeptides for in vivo pharmaceutical use. Accordingly, mimetics of F3 and/or NB-3 and complexes thereof may be designed, especially for pharmaceutical use. Typically a mimetic of one or both of F3 and NB-3 will be capable of binding to a Notch  
15 molecule, preferably the extracellular domain of Notch 1 or 2, to mimic the effects of that protein or proteins binding to the same molecule.

- The designing of mimetics to a known pharmaceutically active  
20 compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are unsuitable active agents for  
25 oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property.

- 30 There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying  
35 the amino acid residues in the peptide, e.g. by substituting each



residue in turn. Alanine scans of peptide are commonly used to refine such peptide motifs. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

5

Once the pharmacophore has been found, its structure is modelled according to its physical properties, eg stereochemistry, bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR.

10 Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

15 In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this in the design of the mimetic.

20

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to

25 synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. Alternatively, where the mimetic is peptide based, further stability can be achieved by cyclising the peptide, increasing its rigidity. The mimetic or mimetics found  
30 by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

In the present case, peptide mapping studies may be used to identify the minimal portion of either F3 or NB-3 required to interact with Notch. This peptide may then be used as a lead compound for mimetic design, as described above.

5

### Antibodies

As antibodies can be modified in a number of ways, the term "antibody" should be construed as covering any specific binding substance having an binding domain with the required specificity. Thus, this term covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that fragments of a whole antibody can perform the function of binding antigens. Examples of binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')<sub>2</sub> fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or

multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993).

Diabodies are multimers of polypeptides, each polypeptide  
5 comprising a first domain comprising a binding region of an immunoglobulin light chain and a second domain comprising a binding region of an immunoglobulin heavy chain, the two domains being linked (eg by a peptide linker) but unable to associate with each other to form an antigen binding site: antigen binding  
10 sites are formed by the association of the first domain of one polypeptide within the multimer with the second domain of another polypeptide within the multimer (WO94/13804).

Where bispecific antibodies are to be used, these may be  
15 conventional bispecific antibodies, which can be manufactured in a variety of ways (Holliger, P. and Winter G. Current Opinion Biotechnol. 4, 446-449 (1993)), eg prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. It may be preferable to use scFv  
20 dimers or diabodies rather than whole antibodies. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction. Other forms of bispecific antibodies include the single chain "Janusins" described in Traunecker et al, Embo  
25 Journal, 10, 3655-3659, (1991).

It may be desirable to "humanise" non-human (eg murine) antibodies to provide antibodies having the antigen binding  
30 immunogenic response of the antibodies, eg when they are used in human therapy. Thus, humanised antibodies comprise framework regions derived from human immunoglobulins (acceptor antibody) in which residues from one or more complementary determining regions (CDR's) are replaced by residues from CDR's of a non-human  
35 species (donor antibody) such as mouse, rat or rabbit antibody

having the desired properties, eg specificity, affinity or capacity. Some of the framework residues of the human antibody may also be replaced by corresponding non-human residues, or by residues not present in either donor or acceptor antibodies.

- 5 These modifications are made to further refine and optimise the properties of the antibody.

Aspects and embodiments of the second aspect of the present invention will now be illustrated, by way of example, with  
10 reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

15 **Brief Description of the Figures Relating to the Second Aspect of the Present Invention**

**Figure 1.** Adhesion of OLN-93 cells or mouse Notch1 transfected Hela cells with or without antibody blocking treatment to spots  
20 of proteins, such as F3-Fc (a, k-l, and q), CHL1-Fc (a) and NB-3-His (b, e-j, m-p, and r), applied to nitrocellulose was determined. Bars (a-b and q-r) represent the number of adherent cells (mean  $\pm$  SD) from at least three independent experiments. Bar marked \* is highly significantly ( $P < 0.05$ ) different from the  
25 control.

Adhesion of OLN-93 cells on coated F3-Fc and NB-3-His substrates.  
a. Effect of antibody blockade on OLN-93 cell adhesion to F3-Fc substrate. F3: F3 protein substrate only (as a control); CHL1: CHL1 protein substrate only; Anti-F3 or Anti-NB-3: addition of  
30 these two antibodies, respectively, to block the F3-Fc coated substrates before plating OLN-93 cells; Anti-Notch1, Anti-Notch2 or Serum: pre-treated OLN-93 cells with these two specific blocking antibodies or pre-immune serum respectively before plating upon a F3-Fc coated substrate.

- b. Effect of antibody blockade on OLN-93 cell adhesion to NB-3 substrate. NB-3: NB-3.His protein substrate only (as control); Anti-NB-3 or Anti-F3: addition of these two antibodies, respectively, to block the NB-3-His coated substrates before  
5 plating OLN-93 cells; Anti-Notch1, Anti-Notch2 or Serum: pre-treated OLN-93 cells with these two specific blocking antibodies or pre-immune serum respectively before plating upon a NB-3-His coated substrate.
- c-d: Immunofluorescence micrographs of OLN-93 cells stained using  
10 anti-Notch1 antibody (c) and anti-Notch2 antibody (d). Cell surface staining is present in both instances.
- e-j: Bright-field micrographs of OLN-93 cells upon contact with coated NB-3-His substrate after 0.5 hour in culture (e) and in the presence of blocking antibodies against Notch1 (f), NB-3 (g),  
15 Notch2 (h), F3 (i), and pre-immune serum (j). Scale bar in (j): 8  $\mu$ m for (c-j).
- Adhesion of mouse Notch1 transfected Hela cells on coated F3-Fc and NB-3-His substrates.
- k-p: Bright-field micrographs of mock-transfected Hela cells (k)  
20 and mouse Notch1 transfected Hela cells (l-p) upon contact with F3-Fc (k-l) or NB-3-His (m-p) and in the presence of blocking antibodies against NB-3 (n), Notch1 (o), and pre-immune serum (p). Scale bar in (p): 8  $\mu$ m for (k-p).
- q. Effect of antibody blockade on mouse Notch1 transfected Hela  
25 cells interaction with F3-Fc substrate. F3: F3-Fc coated substrate only; Anti-F3: addition of these antibodies to block the F3-Fc coated substrates before plating mouse Notch1 transfected Hela cells; Anti-Notch1 or Serum: pre-treated mouse Notch1 transfected Hela cells with these specific blocking  
30 antibodies or pre-immune serum respectively before plating upon a F3-Fc coated substrate; Hela cells: plating mock-transfected Hela cells upon a F3-Fc coated substrate (as a control).
- r. Effect of antibody blockade on mouse Notch1 transfected Hela  
35 cells adhesion to NB-3-His coated substrate. NB-3: NB-3.His coated substrate only; Anti-NB-3: addition of these antibodies to

block the NB-3.His coated substrates before plating mouse Notch1 transfected Hela cells; Anti-Notch1 or Serum: pre-treated mouse Notch1 transfected Hela cells with these specific blocking antibodies or pre-immune serum respectively before plating upon a NB-3.His coated substrate; Hela cells: plating mock-transfected Hela cells upon a NB-3.His coated substrate (as a control).

**Figure 2.** Biochemical and cellular analysis of the interaction between Notch and F3/NB-3.

- a-d: Reciprocal association of both F3 and NB-3 with Notch1 and Notch2. Lysates of rat brain were analyzed by co-immunoprecipitation with these four antibodies and beads and nonimmune IgG (as controls). In each case, lanes correspond to antibodies as marked (Anti-N1: Anti-Notch1 antibody, Anti-N2: Anti-Notch2 antibody). Western blots were probed with antibodies against F3 (a), NB-3 (b), Notch1 (c) and Notch2 (d).
- e: Schematic diagram of the Notch1 molecule showing the terminology assigned to each subcloned fragment.
- f-g: Coomassie Brilliant blue staining (f) and immunoblot analysis (g) of the four fragments are shown. The anti-Notch1 antibody specifically recognizes N1.3 and N1.4.
- h-i: Analysis of the interaction between Notch1 fragments with F3/NB-3 by using a pull down assay. Rat brain lysates were incubated with GST or GST-fusion proteins (N1.1, N1.2, N1.3, N1.4) bound to Sepharose 4B beads. Bound proteins were eluted with SDS sample buffer and analysed by SDS-PAGE and Western blotting with antibodies against F3 or NB-3.
- j-l: Adhesion of F3-transfected CHO cells (j), NB-3-transfected CHO cells (k), and mock-transfected CHO cells (l) to four different Notch1 fragments and GST. The protein fragments N1.1, N1.2, N1.3 and N1.4, together with GST as control were coated onto surfaces of petri dishes and F3-transfected CHO cells (j), NB-3-transfected CHO cells (k) and mock-transfected CHO cells (l) were plated and maintained in chemically defined medium for 2 hours. Bars represent the number of adherent cells (mean  $\pm$  SD)

from at least three independent experiments. Bar marked \* is highly significantly ( $P < 0.05$ ) different from the control (GST).

**Figure 3.** Western blot analysis of expression of MAG and PLP in co-culture of F3-transfected CHO cells and OLN-93 cells.  
 5 a: Immunoblot analysis of MAG in rat brain homogenates (BRAIN) and cell co-culture extracts. b: Immunoblot analysis of PLP in cell co-culture extracts. OLN: OLN-93 cell culture only; F3/OLN: co-culture of OLN-93 cells and F3-transfected CHO cells; CHO/OLN: co-culture of OLN-93 cells and mock-transfected CHO cells;  
 10 TAG/OLN: co-culture of OLN-93 cells and TAG-1-transfected CHO cells; TAX/OLN: co-culture of OLN-93 cells and TAX-transfected CHO cells.

**Figure 4.** Immunofluorescence localization of Jagged1 and NB-3.  
 15 a-c: At P2, hardly any NB-3 staining was detectable. Jagged1 staining was present as linear streaks consistent with an axonal localization. Scale bar in a-c: 20  $\mu$ m for a-i.  
 d-f: At P5, NB-3 can be observed to cluster at paranodal  
 20 locations. Significantly, there is a distinct boundary between Jagged1 and NB-3 immunofluorescence, best seen in the enlarged images j-o. Scale bar in j-l: 2  $\mu$ m for j-o.  
 g-i: At P14, the distribution of NB-3 and Jagged1 remains unchanged from the P5 pattern, apart from the fact that axon  
 25 density and hence numbers of paranodes have increased.

**Figure 5.** The schematic diagram of the molecular constituents of the paranode in the central nervous system.  
 At this location, multiple oligodendroglial cytoplasmic loops  
 30 (here pictured only as a single loop) intimately contact the axolemma. The present study has revealed that in addition to axonal F3/ contactin and Caspr and glial neurofascin 155 (NF-155), other members of the axoglial junction include axonal NB-3 and glial Notch. It has also been demonstrated that a functional

signalling interaction exists between F3/ NB-3 and Notch. (N: Node of Ranvier, PN: Paranode, JPN: Juxtaparanode).

**Table I**

- 5 Primary rat oligodendrocytes were plated on coated F3-Fc, NB-3-His or BSA (control) substrates, respectively. After 2 hours in culture, total RNA was extracted and subjected to real time RT-PCR analysis of MAG and PLP mRNA expression levels. Relative expression levels were derived using the comparative  $C_T$  method.  
10 ( $C_T$ : cycle threshold).

**Figure 6. Notch and F3 are binding partners.**

- (A) Cell adhesion assay. OLN cells were labeled with  $\alpha$ -Notch1 (a) or  $\alpha$ -Notch2 (b). OLN cells were plated on dishes spotted with F3 (c, e-i) or CHL1 (d). Cells were untreated or pre-treated prior to plating with  $\alpha$ -Notch1 (e) or  $\alpha$ -Notch2 (f), pre-immune serum (serum) (g), or with antigen-depleted  $\alpha$ -Notch1 (D- $\alpha$ -Notch1) (h) or  $\alpha$ -Notch2 (D- $\alpha$ -Notch2) (i). Dotted lines depict the edges of the protein-Fc spots. Adherent cells were visualized by staining with Coomassie Blue. j: Quantification of OLN cell adherence to F3 substrate and the effects of blocking antibodies. #  $p < 0.05$  compared with CHL1, \*  $p < 0.05$  compared with pre-immune serum. Scale bar in (i): 20  $\mu$ m for a, b; 120  $\mu$ m for c-i.
- 20 (B) Cell repulsion assay. mN1-transfected HeLa cells (a) or mock-transfected HeLa cells (b) were plated on F3 coated dishes. Adherent cells were stained with Coomassie Blue. c: Quantification of HeLa cell adherence to F3 and the effects of blocking antibodies. In some experiments, mN1-transfected HeLa cells were pretreated with  $\alpha$ -F3 or  $\alpha$ -Notch1, or with pre-immune serum (serum). #  $p < 0.05$  compared with mock-transfected HeLa cells, \*  $p < 0.05$  compared with pre-immune serum. Scale bar in (b): 15  $\mu$ m for a, b. Bar graphs (Aj, Bc) represent the number of  
25  
30 adherent cells (mean  $\pm$  SD).



**Figure 7.** Notch and F3 associate as a protein complex.

**(A)** F3 co-immunoprecipitates with Notch1 or Notch2. **a:**

Immunoprecipitates from rat brain lysate were prepared using  $\alpha$ -Notch1,  $\alpha$ -Notch2, non-immune IgG or unconjugated beads, and were  
 5 probed with  $\alpha$ -F3. **b:** Reciprocal assays used  $\alpha$ -F3 to capture the protein complex, followed by immunoblotting with  $\alpha$ -Notch1 or  $\alpha$ -Notch2 to detect the binding partner.

**(B)** Subcloning of the Notch1 extracellular domain. **a:** Schematic diagram of Notch1 and its subcloned fragments. **b, c:** Coomassie

10 Blue staining and  $\alpha$ -Notch1 immunoblot of the four fragments, respectively.

**(C)** F3 binds to specific domains of Notch1. **a:** The GST-Notch1 extracellular fragments (N1.1, N1.2, N1.3 and N1.4) or GST alone were used in a GST pull-down assay with rat brain lysate. The  
 15 precipitates and rat brain lysate (right lane) were probed for F3. **b:** Quantification of mock- and F3-transfected CHO cells adhering to culture dishes coated with the four GST fusion fragments or GST alone. Bars represent the number of adherent cells (mean  $\pm$  SD). \*  $p < 0.05$  compared with GST.

20 **(D)** Lipid raft analysis. F3 was mainly localized to the fifth fraction while Notch1 was enriched in fractions 9-12. Caspr was used as a positive control to mark lipid raft fractions. H: Total homogenate.

25 **Figure 8.** NICD translocation.

**(A)** F3-induced NICD nuclear translocation. mNotch1-myc transfected OLN cells were treated with 11.2 nM F3 (**a**), Jagged1 (**b**), BSA (**c**) or pre-incubated with  $\alpha$ -Notch1 EGF (11-12) prior to F3 treatment (**d**) and then stained with  $\alpha$ -NICD. **e:** Quantification

30 of cells with nuclear staining of NICD after treatment with increasing concentrations of F3 and Jagged1. Data are mean  $\pm$  SEM. **f:** OLN cells were cultured with Jagged1-, F3- or mock-transfected

CHO cells, and lysates were immunoprobed with  $\alpha$ -Notch1,  $\alpha$ -Notch2 and  $\alpha$ -tubulin.

- (B) F3-induced RIP was carried out by  $\gamma$ -secretase at the S3 site. OLN cells were pre-treated with 200  $\mu$ M  $\gamma$ -secretase inhibitor, stimulated with F3 (a) or Jagged1 (b), and stained with  $\alpha$ -NICD. After treatment with F3 (c, d) or Jagged1 (e, f), myc-tagged V1744K or V1744L mutant-transfected OLN cells were immunolabeled for c-myc. g:  $\alpha$ -c-myc immunoprecipitates from OLN cells expressing myc-tagged wild-type Notch1 or V1744K and V1744L mutants were immunoblotted with  $\alpha$ -NICD (which recognizes both the ~300 kDa full-length and the ~120 kDa intracellular portion of Notch1) or  $\alpha$ -V1744 (which only recognizes NICD after cleavage at the S3 site). Scale bar in (Bf): 15  $\mu$ m for Aa-d, Ba-f.
- (C) Upregulation of Notch1 and Notch2. Total (cytoplasmic plus nuclear) NICD staining intensity was quantified in F3-treated and BSA-treated mNotch1-myc transfected OLN cells (a). OLN cells cultured alone or with F3-, mock, TAG-1-, or TAX-transfected CHO cells were lysed and probed with  $\alpha$ -Notch1,  $\alpha$ -Notch2 and  $\alpha$ -Notch3 (b). c: Real-time PCR assay of Notch mRNA levels in OLN cells treated with 11.2 nM F3, Jagged1 or PBS. Notch mRNA levels were normalized to  $\beta$ -actin. Bars are mean  $\pm$  SEM. \* $p$ <0.05 compared with PBS.

- Figure 9.** OLN cellular processes halt and alter their morphology upon contact with F3-transfected CHO cells. Cellular processes (arrows in a and b) of OLN cells extend towards F3-transfected CHO cell somata and upon contact with them, terminate and elaborate a flattened cytoplasmic sheet that envelops the cell body. This phenomenon is indicated by asterisks (\*). a: Both OLN and F3-transfected CHO cells were pre-stained with PKH26 red fluorescent dye. b: The bright-field micrograph corresponding to (a). c, f, i: OLN cells were pre-stained with PKH26 red fluorescent dye. d, g, j: Both OLN cells and F3-

transfected CHO cells were stained for c-myc (green). e, h, k: Merged images of (c, d), (f, g), (i, j), respectively. l-q: In the control systems, cellular processes (arrowheads) of OLN cells extend past transfected CHO cell bodies. (l, m), (n, o) and (p, q) are corresponding PKH26 red fluorescent and bright-field micrographs of co-cultures of OLN cells with mock- (l, m), TAX- (n, o) and TAG-1- (p, q) transfected CHO cells. r: Quantification of OLN cellular processes extending past transfected CHO cell bodies in the co-cultures. Data are mean  $\pm$  SD. \*  $p < 0.01$  compared with controls. Scale bar in (q): 25  $\mu$ m for (a,b,l-q) and 15  $\mu$ m for (c-k).

**Figure 10.** MAG is upregulated by F3/Notch interaction.

(A) MAG is upregulated by F3. CHO cells and transfected derivatives do not express classic ligands of Notch, Delta, Jagged1 and Jagged2 (a). Lysates of rat brain, OLN alone, and the indicated co-cultured cells were probed with  $\alpha$ -MAG (upper panel) or  $\alpha$ - $\gamma$ -tubulin (bottom panel) (b). (c) Measured by real-time PCR, MAG mRNA in primary OLs is elevated significantly by F3, versus BSA treatment. The raw data were normalized to GAPDH using comparative  $C_t$  method.

(B) F3, but not Jagged1, upregulates MAG. mNotch1-myc transfected OLN cells were treated with 11.2 nM F3-Fc (a, d) or Jagged1 (b, c, e, f) and labeled using  $\alpha$ -MAG (a, b) or  $\alpha$ -CNPase (d, e). The arrows in (b, e) indicate the cell bodies, which can be better viewed in bright-field pictures (c, f). g: Fluorescence intensities of MAG and CNPase staining in cells treated with F3, Jagged1, BSA, or pretreated with  $\alpha$ -Notch1 EGF (11-12) followed by F3. Data are mean  $\pm$  SEM. h: Quantification of the surface area (mean  $\pm$  SEM) occupied by cells treated with F3, Jagged1 or BSA. \* $p < 0.05$ ; \*\* $p < 0.01$  compared with BSA.

(C) MAG upregulation is F3/Notch interaction-dependent.

a-j: OLN cells transfected with Notch ICD-deleted mutants, dn-N1 (a), dn-N2 (b); LacZ (c); S3 cleavage mutants, V1744K (d) and

V1744L (e); Notch ECD-deleted mutants, caN1 (f, g) and caN2 (h, i), were treated (a-e) or untreated (f-i) with 11.2 nM F3 and double labeled for MAG (red) and V5 (a-c, f, h) (green) or c-myc (d, e) (green). The transfected cells in f, h can be better  
 5 viewed as indicated by arrows in bright-field pictures g, i, respectively. j: MAG fluorescence intensities in cells transfected with various indicated constructs followed by different protein treatments. Data are mean  $\pm$  SEM. ECD: extracellular domain; TM: transmembrane domain; ICD: intracellular domain. The S3 site mutations in V1744K and V1744L  
 10 constructs were indicated by triangles in the transmembrane region. \*  $p < 0.01$  compared with F3-treated OLN cells. Scale bar in (Ci): 20  $\mu$ m for (Ba-f); 40  $\mu$ m for (Ca-i).

15 **Figure 11.** MAG expression is independent of *Hes1* and dependent on DTX1.

(A) MAG upregulation is independent of *Hes1* expression. (a) OLN cells were treated with the indicated ligands or compounds. At the times shown, *Hes1* transcripts were quantified using real-time  
 20 PCR and normalized to that at the start of the time course. (b, d, e) OLN cells were untreated or pre-treated with *Hes1* sense (Hes1-S) or antisense (Hes1-AS) oligonucleotides followed by 11.2 nM F3. Cell lysates were probed with  $\alpha$ -Hes1 (upper panel) or  $\alpha$ -nuclear matrix protein (N-matrix) (bottom panel) (b) or cells  
 25 were labeled for MAG (d, e). Also, OLN cells transfected with pGVB/*Hes1* reporter alone or together with constructs expressing caN1, RBP-J, or myc-tagged dn-RBP-J were subjected to luciferase assay (c). Data are mean  $\pm$  SD. f: OLN cells transfected with dn-RBP-J-myc were treated with 11.2 nM F3 and double stained for MAG  
 30 (red) and c-myc (green). g: MAG staining intensity in OLN cells with various treatments indicated above. Data are mean  $\pm$  SEM. \* $p < 0.01$  compared with cells treated with F3 alone.

(B) MAG upregulation involves DTX1. a: DTX1 constructs used in luciferase reporter assays and immunostaining study. N terminal:

N terminal domain; Pro: Proline-rich motif; Ring finger: Ring-H2 finger motif. OLN cells were transfected with pGVB/Hes1 reporter alone or together with indicated expression constructs. **b:** Luciferase reporter activity in these cells. Data are mean  $\pm$  SD.

5 DTX1 (c-e)-, DTX1-D1-HA (f-h)-, or DTX1-D2-Flag (i-k)-transfected OLN cells were treated with 11.2 nM F3 and double labeled for MAG (red) and related tags (green). **l:** MAG fluorescence intensity in OLN cells transfected with indicated constructs followed by different protein treatments. Data are mean  $\pm$  SEM. \*  $p < 0.01$

10 compared with F3-treated OLN cells. Scale bar in (Bk): 30  $\mu$ m for Ac-f, Bc-k.

**Figure 12.** F3/Notch signaling via DTX1 promotes OPC differentiation.

15 Purified Ng2+/CNPase-OPCs (a), were treated with BSA (b), F3 (c) or Jagged1 (d) for 2 days, double labeled for Ng2 (red) and CNPase (green) and counted (k). OPCs were also transfected with tagged dn-N1 (e, h) and DTX1-D2 (f, i), followed by F3 treatment or with caN1 and left untreated (g, j). Cells were double stained

20 for the appropriate tag (green) and CNPase (red; e-g) or Ng2 (red; h-j), and counted (k). Scale bar in (j): 25  $\mu$ m for (a, e-inset), 100  $\mu$ m for (b-j).

**Figure 13.** Proposed model of distinct ligand-dependent Notch signaling pathways during development.

25 F3 interacts with the Notch receptor on the opposing cell surface to stimulate Notch/RBP-J signaling pathway to recruit DTX1 before or after releasing NICD into the cytoplasm. The NICD/RBP-J/DTX1 complex may undergo specific but unidentified modification prior

30 to translocation into the nucleus where it activates target genes such as MAG. This signaling may contribute to OL maturation after P6 when decreased Jagged1 expression favors the initiation of F3/Notch signaling. In contrast, before P6, Jagged1/Notch signaling activates the NICD/RBP-J-dependent transcription of

35 target genes such as *Hes1* and predominantly inhibits OPC

differentiation. ECM: Extracellular matrix; C: Cytoplasm; N: Nucleus; NICD<sup>Jag1</sup>, NICD<sup>F3</sup>: NICD released upon Jagged1 and F3 activation, respectively; E: embryo; P6, P15: postnatal day 6 and 15, respectively; A: adult; OPC: oligodendrocyte precursor cell; 5 O: oligodendrocyte; right bottom cartoon: myelinating oligodendrocyte ensheathing the axon.

**Figure 14.** NB-3 is a paranodal neuronal molecule.

- A. NB-3 is expressed by neurons. Purified neurons, OLs and 10 astrocytes of E17 rats were double stained for NB-3 and corresponding surface marker: NF200 (a), Gal-C (b) and GFAP (c), respectively. Scale bar in (c): 30  $\mu$ m for (a-c).
- (d) NB-3 is expressed from E17. Brain stem from rats with indicated ages were homogenized and subjected to immunoblot for 15 NB-3, F3, MAG.
- (B) NB-3 is localized at the paranode. Brain stem sections from 90 day old rats were double labeled for NB-3 and Caspr (a-c) or NB-3 and sodium channels (d-f). Scale bar in (f): 15  $\mu$ m for (a-f).
- 20 (C) Lipid raft assay. NB3 was enriched in fraction 5, the same fraction as F3 and Caspr. H: Total homogenate.

**Figure 15.** NB-3 is a functional ligand of Notch1.

- (A) NB-3 binds to Notch1. (a) NB-3 co-immunoprecipitated with 25 Notch1. Immunoprecipitates from rat brain lysates using  $\alpha$ -Notch1 and  $\alpha$ -NB-3 were probed with  $\alpha$ -NB-3 or  $\alpha$ -Notch1, respectively. (b) Cell adhesion assay. OLN cells were seeded on NB-3 substrate and adhere to it. Adhesion was specifically blocked by  $\alpha$ -Notch1 or  $\alpha$ -NB-3. #  $p < 0.05$  compared with CHL1; \*  $p < 0.05$  compared with pre-immune serum. (c) NB-3 binds to specific region on Notch1. The 30 Notch1 GST fusion proteins or GST alone were used in a GST pull-down assay from rat brain lysates. The precipitates and brain lysates were probed for NB-3. (d) Quantification of adherent NB-3- and mock-transfected CHO cells to the four Notch1 GST fusion

fragments. \*  $p < 0.05$  compared with GST. Bar graphs (b, d) represent the number of adherent cells (mean  $\pm$  SD).

(B) NB-3/Notch interaction induces NICD nuclear translocation in OLN cells. mNotch1-myc transfected OLN cells treated with NB-3 (a), Jagged1 (b) and BSA (c) were immunostained for NICD. Some cells were treated with EGF antibody (d) or  $\gamma$ -secretase inhibitor (e) before NB-3 stimulation. OLN cells were also transfected with V1744K-myc (f, h) or V1744L-myc (g, i), treated with NB-3 (f, g) or Jagged1 (h, i), and immunostained with c-myc antibody to locate NICD. Scale bar in (a): 20  $\mu$ m for (a-i). (j) After NB-3 or Jagged1 treatment,  $\alpha$ -c-myc precipitates from mNotch1-myc, V1744K-myc or V1744L-myc transfected OLN cells were immunoblotted by  $\alpha$ -NICD (upper panel) or  $\alpha$ -V1744 (lower panel).

(C) *Hes1* and *Hes5* are not activated by NB-3. OLN cells treated with NB-3 for different durations as indicated were lysed and the extracted mRNA subjected to real-time PCR (a). The data were normalized to the mRNA level at the starting point. Other cells were treated with PBS, BSA, L1 or NB-3 for 48 hours and analysed by real-time PCR (b). (c) OLN cells were transfected with *Hes1* or *Hes5* luciferase reporter alone followed by NB-3 treatment or with can1 construct. 24 hours post-transfection, cells were subjected to luciferase assay. Data are mean  $\pm$  SD.

**Figure 16.** NB-3/Notch interaction upregulates MAG via DTX1.

(A) MAG was upregulated in the co-culture of OLN-93 cells and NB-3-transfected CHO cells (a). N-matrix: nuclear matrix protein. CHO/OLN, NB3/OLN: co-culture of OLN-93 cells and mock- or NB-3-transfected CHO cells, respectively. (b) MAG mRNA in primary OLN increased about 24 fold after NB-3 stimulation as monitored by real-time PCR. GAPDH was used as an internal control. OLN cells were treated with NB-3 (c), Jagged1 (d, e) and BSA (not shown) and immunostained for MAG. The fluorescence intensity of MAG was counted (f). Data are mean  $\pm$  SEM.

(B) NB-3-induced MAG upregulation involves DTX1. OLN cells were transfected with dn-N1-V5 (a), V1744K-myc (b), V1744L-myc (c), caN1 (d, e), dn-RBP-J-myc (h), DTX1-myc (i), DTX1-D1-HA (j), and DTX1-D2-Flag (k), and treated with NB-3. The cells were then immunostained for MAG and corresponding tag. The fluorescence intensity of MAG was counted in transfected and non-transfected cells (l). Data are mean  $\pm$  SD. (f) Schematic structure of DTX1 and its two deletion mutants. Number 1, 2, 3 correspond to N-terminal, proline-rich region and Ring-H2 finger motif, respectively. (g) Hes1 luciferase reporter assays to confirm the validity of indicated constructs. Data are mean  $\pm$  SD. The scale bar in (k): 30  $\mu$ m for (Ac-e, Ba-k).

**Figure 17.** NB-3 developmentally clustering at paranodes promotes OPC differentiation via Notch1/DTX1 signaling pathway. (A) NB-3 and Jagged1 are distinctly distributed during development. Brain stems from P2 (a), P5 (b, c) rats were double stained for Jagged1 (green) and NB-3 (red). The scale bar in (c): 30  $\mu$ m for (a, b), 5  $\mu$ m for (c). (B) NB-3/Notch accelerates OPC differentiation via DTX1. Purified Ng2+ OPCs from P7 rat optic nerve (a) were treated with BSA (b), NB-3 (c) or Jagged1 (d) for 2 days and double labelled for Ng2 (red) and CNPase (green). Other cells were transfected with dn-N1 (e), DTX1-D1 (f) followed by NB-3 stimulation or caN1 (g) alone. Cells were then immunostained for tags (green) and CNPase (red). The percentage of CNPase+ cells were counted (h). Data are mean  $\pm$  SEM. The scale bar in (g): 40  $\mu$ m for (a-g).

**Figure 18.** NSC: Rat brain stems of indicated ages were subjected to Western blot for NB-3 and Notch1 expression patterns.

**Figure 19.** NSC: NB-3 is a functional ligand of Notch1. (A) NSCs express Notch1. NSCs were double stained for precursor marker nestin (a) and Notch1 (b). (c) is the merged picture. Scale bar in (c): 60  $\mu$ m for (a-c).



- (B) NB-3 binds to Notch1. P0 rat brain samples were precipitated by Protein A beads coupled with NB-3-Fc fusion protein,  $\alpha$ -Notch1 or  $\alpha$ -NB-3 and the precipitates were blotted as indicated (a). N1-transfected Hela cells (N1) were seeded onto coated NB-3 substrate in the absence (b) or presence of blocking antibodies:  $\alpha$ -NB-3 (c),  $\alpha$ -Notch1 (d) or pre-immune serum (s) (e). The adherent cells were counted (f). Data are mean  $\pm$  SD. \*  $p < 0.05$  compared with mock-transfected Hela cells; #  $p < 0.05$  compared with pre-immune serum.
- 10 (C) NB-3 induces NICD nuclear translocation. NSCs were individually treated with NB-3 (12.5nM) (a), Jagged1 (50nM) (b) or BSA (c) for 24 hours then fixed and triple stained for nestin (green), NICD (red) and Hoechst 33258 (blue) to locate NICD. Scale bar in (c): 20  $\mu$ m for (a-c).
- 15 (D) NB-3 does not activate Hes1. NSCs were transfected with Hes1 luciferase reporter construct followed by NB-3 or Jagged1 treatment or cotransfected with caN1. 24 hours post-transfection, the cells were lysed and subjected to luciferase assays (a). Other NSCs were transfected with pE7 luciferase reporter together
- 20 with indicated constructs with or without NB-3 treatment and subjected to luciferase assays (b). Data are mean  $\pm$  SD.

**Figure 20. NSCs: NB-3 promotes OL generation.**

NSCs were passaged into mitogen-withdrawn culture medium that was supplemented with NB-3 (a, d), BSA (b, e) or Jagged1 (c, f). After 7 DIV differentiation, the cells were triple stained for CNPase (a-c, red) or  $\beta$ -tubulin (d-f, red), GFAP (a-f, green) and Hoechst 33258 (a-f, blue). Other NSCs were individually immunolabelled with marker antibody and subjected to flow

30 cytometry. The percent of each type of cells: OLs, neurons and astrocytes were counted (g). Data are mean  $\pm$  SEM. Scale bar in (f): 40  $\mu$ m for (a-f).

**Figure 21.** NB-3/Notch signalling pathway via DTX1 instructs oligodendroglialogenesis.

NSCs were transfected with dn-N1 (a, b), DTX1-D2 (d, e) followed by NB-3 treatment or caN1 (f, g) and double stained for appropriate tags and CNPase (a, d, f) or GFAP (b, e, g). (c) Schematic structure of DTX1 and DTX1-D2 constructs. The validity of the constructs utilized here was confirmed in Hes1 luciferase reporter assays (h). The percents of transfected cells that were positive for CNPase or GFAP were counted (i). Data are mean  $\pm$  SD. Scale bar in (g): 20  $\mu$ m for (a, b, d-g).

**Figure 22.** The putative model.

The extracellular NB-3/Notch interaction releases from the membrane NICD, which recruits DTX1 and translocates into the nucleus where the complex mediates directly or indirectly CNPase expression, thus promoting oligodendroglialogenesis. NSC: neural stem cell; O: OLs; M: membrane; N: nucleus; CNP: CNPase.

#### Detailed Description of the Second Aspect of the Invention

##### **Results**

Notch is the oligodendroglial surface receptor for F3 and NB-3. The inventor set out to identify the glial receptor for F3 and NB-3. Both molecules have a basic structure composed of immunoglobulin and fibronectin type III repeats. Previous work investigated the effect of F3/tenascin-R (TN-R) interaction in various in-vitro models (Xiao et al, 1996, 1997, and 1998 all incorporated herein by reference). Of relevance to the present study, the inventor had demonstrated that the epidermal growth factor-like repeats of TN-R constituted the binding site for its neuronal receptor F3. The extracellular domain of Notch is composed primarily of epidermal growth factor-like repeats, hence making it a plausible candidate as the glial receptor of F3 and NB-3. In addition to this structural factor, the temporal and spatial location of Notch on the maturing oligodendrocyte in

contact with the axon additionally attests to its suitability as a receptor for paranodal F3 and NB-3. As the experiments utilized the oligodendrocyte cell line OLN-93, it was confirmed using immunocytochemistry that these cells indeed express both Notch1 and Notch2 on their surface (Fig. 1c-d).

The inventor first carried out substrate adhesion assays to determine if F3 and NB-3 could be binding partners of Notch. To do this, the OLN-93 cells were cultured on F3 and NB-3 protein substrates in the presence or absence of respective blocking antibodies against F3, NB-3 and Notch. The results show that OLN-93 cells adhered readily to F3 and that the adhesive effects were blocked by antibodies against the F3 substrate and both Notch1 and Notch2 (Fig. 1a-j). Cell adhesion to the F3 substrate was also reduced when NB-3 antibodies were added. The OLN-93 cells also adhered to NB-3 and incubation with antibodies against NB-3, Notch1 and Notch2 reduced cellular adhesion. In addition, when OLN-93 cells were plated upon NB-3, they rapidly underwent a marked morphological change. They enlarged and transformed into oval-shaped to circular flattened cells characterized most strikingly by an expansive cytoplasmic sheet (Fig. 1c-j). This alteration in morphology also occurred when OLN-93 cells were plated upon the F3 substrate, but only after a longer duration (not shown). As controls, the inventor used bovine serum albumin and CHL1 (Holm et al, 1996), another neural cell adhesion molecule of the immunoglobulin superfamily, but neither substrate promoted OLN-93 cell adhesion. This suggests that the F3/NB-3 signal may constitute a mechanism that triggers oligodendrocyte differentiation.

Additional evidence for the interaction between F3/ NB-3 and Notch was provided using the same assay system and blocking antibodies, but using mouse Notch1-transfected HeLa cells instead of OLN-93 cells (Fig. 1k-r). In this instance, the inventor noted interestingly that Notch1-transfected HeLa cells were repelled

from F3-Fc and this repulsive effect was reversed when antibodies against F3 and Notch1 were added. In the case of NB-3-His, there was adhesion of the Notch1-transfected HeLa cells, which was inhibited when antibodies against NB-3 and Notch1 were added.

5 These cellular studies strongly suggest that Notch is a receptor for F3 and NB-3.

#### **F3 and NB-3 bind to distinct sites on Notch**

To analyse further the presence of an association between F3 and Notch and between NB-3 and Notch, the inventor carried out several biochemical and molecular approaches (Fig. 2). Rat brain membrane preparations were solubilized in 2% Triton X-100 and were immunoprecipitated with antibodies to Notch1 and Notch2. Western Blot analysis using anti-F3 and anti-NB-3 showed that the anti-Notch1 and 2 antibodies precipitates contained both F3 and NB-3 (Fig. 2a-b). Control immunoprecipitates with non-immune IgG were negative for both F3 and NB-3. In the reverse co-immunoprecipitation experiment, anti-F3 and anti-NB-3 was used to immunoprecipitate similar brain preparations and the blot probed with antibodies to Notch1 and Notch2 (Fig. 2c-d). These co-immunoprecipitation results provide evidence that both F3/ Notch and NB-3/ Notch interactions may underlie the formation of protein complexes in the brain.

25 To allow the inventor to further characterize this interaction, the extracellular domain of Notch1 was arbitrarily divided into four equal sized overlapping 1.5 kb fragments (Fig. 2e) and subcloned each of them in frame into pGEX-KG (Guan and Dixon, 1991) for GST fusion protein production. The four protein fragments were expressed by induction of transformed *E. coli*. The inventor's nomenclature for the four Notch1 protein GST fusion protein fragments is N1.1, N1.2, N1.3 and N1.4, proceeding in a N- to C-terminal direction. Coomassie Blue staining and immunoblots of the four fragments are shown (Fig. 2f-g). The antibody to Notch1 specifically recognized N1.3 and N1.4 (Fig.

2g). Given the inventor's finding that the adhesion of OLN93 cells to F3 and NB-3 were blocked by same Notch1 antibodies, F3 and NB-3 may at least have a common binding site on either N1.3 or N1.4.

5

The inventor then carried out a GST pull-down assay to provide further biochemical evidence that an association exists between Notch and both F3 and NB-3. The four Notch1 GST fusion proteins were used to bind both F3 and NB-3 in a rat brain lysate. Upon  
10 analysis by Western blotting, they discovered that F3 associated with two fragments - N1.1 and N1.3 (Fig. 2h) whilst NB-3 associated with N1.3 only (Fig. 2i). These results serve to refine the inventor's earlier data that F3 and NB-3 are binding partners of Notch and that they have a common binding site on  
15 N1.3.

As an added confirmation of this biochemical interaction, the inventor used the four Notch1 protein fragments to carry out cell adhesion assays in which F3-transfected CHO cells and NB-3-  
20 transfected CHO cells were plated upon each individual fragment. The results support the findings from the GST pull-down assay, in that the F3-transfected cells bound predominantly to N1.1 and N1.3 (Fig. 2j) and the NB-3-transfected cells bound predominantly to N1.3 (Fig. 2k). Altogether, these results provide biochemical  
25 evidence to support the notion that Notch acts as a receptor for F3 and NB-3.

**The expression of MAG is upregulated by the interaction between Notch and F3/NB-3**

30 The above results substantiated the inventor's hypothesis that a molecular interaction occurs between paranodal F3 and NB-3 and oligodendroglial Notch as he has provided evidence of their physical association and also their respective axonal (F3, NB-3) and glial (surface expression of Notch1 on oligodendrocytes)  
35 locations. Next, he explored how this signalling event could be

related to myelination. As the myelinating oligodendrocyte contacts and wraps the axons in a multi-layered spiral sheath, the protein components of the myelin sheath logically become upregulated as the event progresses. Thus using co-cultured OLN-93 cells and F3- or NB-3-transfected CHO cells, the inventor investigated the expression of myelin-specific proteins in these cellular models of F3/ NB-3-Notch interactions. Pure OLN-93 cultures and co-cultures between OLN-93 cells and mock-, TAG- or TAX- transfected cells were used as controls. These cellular cultures were homogenized to prepare membrane extracts and analyzed them by immunoblotting to ascertain the levels of MAG (myelin-associated glycoprotein) and PLP (proteolipid protein), components of the myelin sheath in the CNS. It was shown that when OLN-93 cells were co-cultured with F3-transfected CHO cells or with NB-3-transfected CHO cells (not shown), MAG became specifically upregulated (Fig. 3). PLP levels, however, were the same in all the culture systems investigated. This suggested that the F3/Notch or NB-3/Notch interaction was active in the setting of myelination. To provide further evidence to support this finding, the inventor proceeded to analyze if a similar alteration in myelin-specific proteins occurred when primary oligodendrocytes were employed instead of the OLN-93 cell line. Therefore, primary cultures of rat (postnatal day 1 or 2) oligodendrocytes were prepared and plated them upon F3-Fc and NB-3-His fusion protein substrates. BSA (bovine serum albumin) was used as a control substrate. Again, this direct cell-protein contact strived to simulate the contact between axonal ligands and glia. Two hours after the cells were plated onto the proteins, total RNA was isolated from each of these interacting systems and performed real-time RT-PCR assays to measure the mRNA expression levels of MAG and PLP. It was noticed that in both the systems where primary oligodendrocytes were plated upon F3 and NB-3, MAG expression levels were approximately 8-fold higher than in the control system where BSA was used as a substrate (Table I). Message levels of PLP however were not found to be elevated

when compared with the control. These findings are in agreement with the western blot results and further confirm the presence of myelin-specific gene up-regulation arising as a result of F3/ Notch and NB-3/ Notch signalling.

5

#### **NB-3 and Jagged1 localize to distinct axonal domains**

It has been explained how Jagged1 influences oligodendrocyte differentiation via Notch (Wang et al, 1998). When the oligodendrocyte cellular processes encounter F3 and NB-3 at the paranodes, a separate instruction could be conveyed to the myelinating cell, nevertheless via the same receptor - Notch. Having established that F3 and NB-3 are confined to paranodal regions, it became apparent that if Jagged1 were confined to the axonal segment enclosed by paranodes, namely the internode, a signal switch mechanism at work during axonal ensheathment could exist. The inventor therefore analyzed the distribution of NB-3 and Jagged1 in rat brain stem sagittal cryosections using immunofluorescence (Fig. 4). Three separate age groups were analyzed - postnatal days 2, 5 and 14, thus allowing the axonal pattern of NB-3 distribution to be investigated as the animal matured. The inventors could not observe any staining for NB-3 at P2 (Fig. 4a-c), whereas by P5 (Fig. 4d-f), NB-3 could be seen clustered at paranodes. From this result, the inventors inferred that NB-3 is likely diffusely distributed over the axonal surface initially and then translocates to the paranodes during development to form distinct clusters. Importantly, Jagged1 immunoreactivity was confined to internodes and was separate from paranodes (Fig. 4f). These findings suggest that the Notch receptors on myelinating oligodendrocytes may switch axonal binding partners from Jagged1 to F3/NB-3 when they migrate along the axon from the internodes to adjacent paranodes, thus becoming sequentially exposed to different signals.

**Notch is the oligodendroglial surface binding partner of F3.**

Axonal F3 congregates at the paranode, a potential site for F3 to interact with myelinating glia (Girault and Peles, 2002). Notch is a plausible binding partner since its extracellular portion possesses many EGF-like repeats (Martinez Arias et al, 2002) and is abundantly expressed on maturing OLs (Lardelli et al, 1994; Wang et al, 1998). To investigate this potential interaction an OL cell line OLN-93 (OLN) was utilized. OLN cells were derived from spontaneously transformed cells in rat brain glial cultures and resemble maturing OLs (Richter-Landsberg and Heinrich, 1996). OLN cells no longer express the progenitor cell surface marker A2B5, and are positive for only one isoform of myelin basic protein (MBP) (~ 14 kDa), characteristic of immature OLs. Immunocytochemistry confirmed that OLN cells express Notch1 and Notch2 on their surface (Fig. 6Aa, b).

To investigate if F3 could bind to Notch, cell adhesion assays were performed as described (Xiao et al, 1996). OLN cells were plated on F3-Fc (F3) substrate in the absence or presence of blocking antibodies. OLN cells adhered readily to F3 (Fig. 6Ac), but not to CHL1-Fc (CHL1), another neural cell adhesion molecule (Holm et al, 1996) (Fig. 6Ad). Adhesion was blocked by pre-incubation with F3 (Fig. 6Aj), Notch1 or Notch2 antibodies (Fig. 6Ae, f, j), but not by pre-immune serum or antigen-depleted antibodies to Notch1 or Notch2 (Fig. 6Ag, h, i). Murine Notch1 (mN1)-transfected HeLa cells (Logeat et al, 1998) were also used in cell repulsion assays, another approach to investigate ligand-receptor relationships (Fig. 6B). mN1-transfected HeLa cells were repelled from F3 (Fig. 6Ba) compared with mock-transfected HeLa cells (Fig. 6Bb). Repulsion was reversed by pre-treating the cells with F3 or Notch1 antibodies, but not with pre-immune serum (Fig. 6Bc). These studies suggest that Notch1 interacts with F3.

#### **F3 binds to specific sites on Notch1**

To confirm F3/Notch interaction, rat brain membrane samples were immunoprecipitated with Notch1 or Notch2 antibodies.



Immunoblotting of the precipitates using F3 antibody showed that they contained F3 (Fig. 7Aa). In a reciprocal assay, an F3 antibody-precipitate was probed with Notch1 or Notch2 antibodies (Fig. 7Ab). These results indicate that Notch and F3 can form complexes.

To identify the specific site(s) on Notch1 for F3 binding the mouse Notch1 extracellular domain was divided into four equal-sized fragments termed N1.1, N1.2, N1.3 and N1.4 (Fig. 7Ba) and produced them as recombinant GST fusion proteins as identified (Fig. 7Bb, c). The Notch1 antibody used in the aforementioned cellular studies recognized N1.3 and N1.4, but not N1.1 or N1.2. GST pull-down assays with rat brain lysates revealed that F3 associated with N1.1 and N1.3 (Fig. 7Ca). To confirm this, F3-transfected CHO cells (Gennarini et al, 1991) were seeded onto culture dishes coated with the four GST fusion proteins. Cells bound predominantly to N1.1 and N1.3. Mock-transfected CHO cells did not bind (Fig. 7Cb).

#### **F3 and Notch1 are not co-localized in lipid rafts**

F3 is a surface molecule localized in lipid rafts of OLS (Krämer et al, 1999). To ascertain whether F3/Notch interaction could occur in cis in these microdomains, they were isolated from P15 rat cerebral cortex. Both F3 and Caspr were detected in fraction 5 of the sucrose density gradient (Fig. 7D) as reported (Faivre-Sarrailh et al, 2000). Notch1 was found only in fractions 9-12 that are enriched in cytoskeleton-associated proteins (Fig. 7D). The same results were obtained using adult rat cerebral cortex (not shown). Thus, F3 and Notch1 are unlikely to complex laterally within lipid rafts. Altogether, these observations suggest that F3 is a trans-binding partner of Notch.

**NICD translocates to the nucleus after Notch interacts with F3**  
The immediate consequence of Notch activation is the release and transport of NICD to the nucleus (Schroeter et al, 1998). To

determine if F3 acts as a functional ligand to initiate these events, myc-tagged full-length mouse Notch1 (mNotch1-myc) was transfected into OLN cells. Cells were treated with different proteins and immunolabeled for NICD using NICD antibody (Logeat *et al*, 1998). In F3-treated cells concentrated NICD staining was observed mainly in the nuclei (Fig. 8Aa), similar to Jagged1-induced NICD translocation (Fig. 8Ab). BSA failed to trigger this event (Fig. 8Ac). Pre-incubation with antibody to Notch1 EGF repeats 11-12 (EGF 11-12 antibody, which crossreacts with Notch2, not shown) abolished F3-induced NICD translocation (Fig. 8Ad). Brefeldin A and monensin (not shown), compounds that inhibit the membrane insertion of Notch1 (Schroeter *et al*, 1998), also prevented NICD translocation. Cell treatment with increasing concentrations of F3 or Jagged1 led to a similar increase in nuclear clustering of NICD, indicating that translocation occurs in an F3 or Jagged1 concentration-dependent manner (Fig. 8Ae). OLN co-cultured with either F3- or Jagged1-transfected CHO cells, but not with mock-transfected CHO cells, also resulted in production of Notch2 intracellular domain (ICD) (Fig. 8Af). Notch2 ICD antibody was not crossreactive with Notch1 ICD (not shown). These results demonstrate that F3, like Jagged1, can activate Notch1 and Notch2, leading to subsequent nuclear translocation of NICD.

**F3 induces Notch intramembrane cleavage at the S3 site.**

As a prerequisite for activation, Notch undergoes RIP at the S3 site (V1744) by the presenilin-dependent  $\gamma$ -secretase (Schroeter *et al*, 1998; Huppert *et al*, 2000). To clarify the nature of F3-induced cleavage, mNotch1-myc transfected OLN cells were pre-incubated with  $\gamma$ -secretase inhibitor and then treated with F3 or Jagged1. In both cases, no NICD staining was observed in the nuclei (Fig. 8Ba, b). Moreover, two S3 cleavage mutants: V1744K-myc and V1744L-myc, which showed reduced proteolysis and parallel reduction in activity (Schroeter *et al*, 1998), were transfected into OLN cells that were then treated with F3 or Jagged1. Cells

showed c-myc immunostaining mainly in the cytoplasm and on the cell surface, but not in the nuclei (Fig. 8Bc-f). In immunoprecipitation assays, c-myc antibody-precipitates from F3- or Jagged1-treated V1744K-myc and V1744L-myc transfected OLN cells could only be labeled with NICD antibody that also recognizes full-length Notch1 (300 kDa) (Fig. 8Bg, upper panel), indicating that these mutant Notch1 molecules remained intact. Only the precipitates from F3- or Jagged1-treated mNotch1-myc transfected OLN cells showed a reactive band upon probing with V1744 antibody that solely recognizes NICD released from S3 (120 kDa) (Fig. 8Bg, lower panel). Altogether, these observations suggest that F3 induces RIP at the Notch1 S3 site.

**F3/Notch interaction upregulates Notch1 and Notch2 expression**

F3, but not BSA, induced a two-fold increase in nuclear NICD (Fig. 8Ca), while there was no noticeable change in cytoplasmic NICD, suggesting that F3 upregulates Notch expression. To investigate this, OLN cells were cultured with mock-, F3-, TAG-1- or TAX-transfected CHO cells. TAG-1 and TAX are members of F3 subfamily (Tsiotra et al, 1993). Expression of Notch1 and Notch2, but not Notch3, increased when OLN cells were cultured with F3-transfected CHO cells (Fig. 8Cb). Real-time PCR confirmed that soluble F3 increased Notch1 and Notch2, but not Notch3 transcripts (Fig. 8Cc), while Jagged1 only upregulated Notch1, but not Notch2. Thus, F3/Notch interaction may provide a feedback loop to specifically upregulate Notch1 and Notch2.

**Oligodendroglial processes alter their morphology upon contact with F3**

To model the scenario of axoglial contact during myelination, the morphology of OLN processes was studied when they contact cell surface-expressed F3. Since OLN cells extend longer processes than primary OLs they are ideally suited for observing subtle morphological changes that occur during contact. F3-transfected CHO cells mimicked the paranodal axonal component. Mock-, TAG-1-

and TAX-transfected CHO cells were used as controls. Remarkably, most OLN processes terminated upon contact with F3-transfected CHO cells and flattened to form a cytoplasmic sheet spreading over the surface of CHO cells, as if in an attempt to envelop the cell (Fig. 9a-k). But this was not observed with mock- (Fig. 9l, m), TAG-1- (Fig. 9n, o) or TAX-transfected (Fig. 9p, q) CHO cells. While approximately 80% of extending processes halted upon reaching F3-transfected CHO cells, with other CHO cells the proportion was only 20% (Fig. 9r). These results show that a signal inducing the morphological change is presented to the oligodendroglial processes when F3 is encountered.

#### **F3, but not Jagged1, upregulates MAG**

To explore how the morphological change described above could relate to F3/Notch signaling, the expression of myelin-associated glycoprotein (MAG) in the aforementioned co-cultures was investigated. Parental and transfected CHO cells did not express Delta, Jagged1 and Jagged2 (Fig. 10Aa). Membrane extracts of co-cultured cells were immunoblotted for MAG. The constitutive level of MAG in OLN cells was very low, if not, undetectable. However, when OLN cells were cultured with F3-transfected CHO cells, MAG was upregulated (Fig. 10Ab). F3-transfected CHO cells do not express MAG (not shown). On the other hand, in real-time PCR, purified primary OLs plated upon F3 substrate showed approximately sixteen-fold increase in MAG transcripts, versus cells seeded on BSA (Fig. 10Ac). OLN cells showed a similar efficiency of MAG upregulation (not shown).

In immunostaining assays, mNotch1-myc transfected OLN cells were immunolabeled for MAG and 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), an OLs specific antigen. Treatment with soluble F3 resulted in a remarkable increase in MAG staining (Fig. 10Ba) and enhanced CNPase staining (Fig. 10Bd), compared with Jagged1 (Fig. 10Bb, c, e, f) or BSA (not shown) treatment. Quantification of MAG and CNPase fluorescence intensities

revealed an approximately 300% increase in MAG and 40% increase in CNPase labeling in F3- versus Jagged1- or BSA-treated cells (Fig. 10Bg). Cell pre-treatment with Notch 1 EGF 11-12 antibody prevented F3-induced increase in MAG and CNPase (Fig. 10Bg),  
5 suggesting that Notch is required for this event. In addition, F3 induced the cells to flatten and form a sheet-like structure (Fig. 10Ba). Quantification of the substratum area covered by cells revealed a two-fold increase in that covered by F3-versus Jagged1- or BSA-treated cells (Fig. 10Bh). These findings confirm  
10 F3-induced MAG upregulation.

#### **MAG is upregulated by F3/Notch interaction**

To better understand the involvement of NICD in this event, OLN cells were transiently transfected with V5-tagged dominant-  
15 negative Notch1 (dn-N1) or Notch2 (dn-N2) (Small et al, 2001), which lack the intracellular regions but can bind to extracellular ligands. Cells were then treated with F3 and double stained with V5 and MAG antibodies. Notably, both dn-N1-V5- (Fig. 10Ca) and dn-N2-V5- (Fig. 10Cb) positive cells were poorly  
20 labeled for MAG. pcDNA4/V5/LacZ (LacZ) was used as a vector control (Fig. 10Cc). Moreover, OLN cells transfected with two S3 mutants, myc-tagged V1744K (Fig. 10Cd) and V1744L (Fig. 10Ce) were treated with F3 and double stained for c-myc and MAG. In either case, F3 failed to upregulate MAG in c-myc-positive cells.  
25 Quantification of MAG fluorescence intensity confirmed that Notch1 or Notch2 dysfunction, in other words, the absence of NICD, abolished F3-induced MAG upregulation (Fig. 10Cj), suggesting that NICD is required for MAG expression.

30 The inductive role of F3 in this event was further investigated by introducing into OLN cells V5-tagged constitutive-active Notch1 (caN1) (Fig. 10Cf, g) and Notch2 (caN2) (Fig. 10Ch, i), which lack the extracellular domains and are ligand-independently active (Small et al, 2001). That is, even in the absence of F3  
35 stimulation, NICD is generated and translocates to the nucleus.

And in OLN cells, caN1 (Fig. 10Ac) and caN2 (not shown) did lead to the transactivation of *Hes1* in luciferase reporter assays. After transfection, cells were double stained for V5 and MAG. Immunolabeling showed that V5-positive cells were faintly stained  
5 for MAG (Fig. 10Cj). Given that Jagged1 also induces NICD release, but does not increase MAG expression, these results demonstrate that MAG upregulation requires F3 induced NICD.

**F3/Notch-induced MAG upregulation is independent of *Hes1***

10 A prominent feature of Notch signaling is the activation of *Hes* genes in an oscillatory manner (Hirata et al, 2002). *Hes1* mRNA expression in OLN cells was thus investigated. In real-time PCR, non-physiological treatment of cells with 2 mM EDTA (Rand et al, 2000) triggered a sharp increase in *Hes1* mRNA during the first  
15 two hours and a return to basal level by three hours (Fig. 11Aa), reflecting the exquisite intrinsic regulation of endogenous *Hes1* expression (Dale et al, 2003). However, F3, Jagged1 or BSA treatment did not significantly alter the baseline oscillating levels of endogenous *Hes1* transcription at short (the first three  
20 hours) or long (12 and 24 hours) times after treatment (Fig. 11Aa).

To investigate whether F3-induced MAG upregulation is related to constitutive levels of *Hes1* protein, *Hes1* antisense  
25 oligonucleotides (Kabos et al, 2002) were used to block basal *Hes1* protein expression in OLN cells (Fig. 11Ab). MAG upregulation was not influenced by this treatment or control sense oligonucleotides (Fig. 11Ad, e, g).

30 RBP-J is a Notch-regulated transcription factor that can activate *Hes* genes (Martinez Arias et al, 2002). OLN cells were transfected with dominant-negative RBP-J-myc (dn-RBP-J-myc), bearing a mutation of lysine 218 to histidine, which abolishes effective high affinity binding to *Hes1* promoter region (Kato et  
35 al, 1997). *Hes1* luciferase reporter assay showed that the mutant

prevented *Hes1* activation by *caN1* (Fig. 11Ac). After transfection, cells were treated with F3 and double labeled for c-myc and MAG. c-myc positive cells showed the same level of MAG as neighboring nontransfected cells (Fig. 11Af, g). These results  
5 indicate that MAG upregulation triggered by F3/Notch signaling is independent of endogenous *Hes1* expression.

#### **F3/Notch-induced MAG upregulation involves DTX1**

Increasing evidence indicates that DTX1 is another downstream  
10 element of the Notch signaling pathway (Martinez Arias et al, 2002). Given that F3-induced MAG upregulation is not related to *Hes1* expression, the role of DTX1 in this event was investigated using myc-tagged wild-type DTX1 (Yamamoto et al, 2001) and its two deletion mutants as depicted (Fig. 11Ba), namely, HA-tagged  
15 DTX1 mutant (DTX1-D1) containing amino acids 1-411 (Yamamoto et al, 2001) and Flag-tagged DTX1 mutant (DTX1-D2) containing amino acids 1-242 (Izon et al, 2002). The two mutants lack the Ring-H2 finger motif, which contributes to DTX1 oligomerization, an essential step for DTX1 functioning (Matsuno et al, 2002). As  
20 previously observed (Yamamoto et al, 2001), *Hes1* luciferase reporter assay showed overexpressed DTX1 inhibited the transactivation of *Hes1* by *caN1* (Fig. 11Bb). On the other hand, both DTX1-D1 and DTX1-D2 restored *Hes1* response to *caN1* (Fig. 11Bb). After transfection, OLN cells were treated with F3 and  
25 double labeled for MAG and corresponding tags. Overexpression of DTX1 had no effect on F3-induced MAG upregulation (Fig. 11Bc-e, 1). Interestingly, MAG upregulation was abolished in the HA-positive or Flag-positive cells (Fig. 11Bf-1). These observations strongly suggest that F3/Notch-induced MAG upregulation involves  
30 DTX1.

#### **F3/Notch signaling pathway via DTX1 promotes OPC differentiation into OLs**

The Jagged1/Notch signaling pathway inhibits differentiation of  
35 OPCs into OLs (Wang et al., 1998). To explore whether F3/Notch

signaling via DTX1 instructs OPC differentiation, purified OPCs positive for the progenitor marker Ng2 (Dawson et al., 2000) (Fig. 12a), were treated with BSA, F3 or Jagged1 for 2 days and then double stained for Ng2 and the OL-specific CNPase (Fig. 12b-d). After F3 treatment, over 70% of OPCs differentiated into CNPase+ OLs compared to ~50% after BSA stimulation, while Jagged1 treatment resulted in nearly all OPCs remaining undifferentiated (Fig. 12k). F3-stimulated cells were more bifurcated and inclined to form a web-like structure (Fig. 12c) than BSA-treated cells (Fig. 12b). Notch and DTX1 involvement was examined by transfecting OPCs with dn-N1-V5 (Fig. 12e, h) and DTX1-D2-Flag (Fig. 12f, i), respectively, followed by F3 treatment for 2 days. Other OPCs were transfected with caN1-V5 and left untreated (Fig. 12g, j). Notably, immunolabeling for tags and CNPase (Fig. 12e-g) or Ng2 (Fig. 12h-j) showed that most dn-N1 (~75%) or DTX1-D2 (~67%) transfected OPCs remained Ng2+ (Fig. 12k), despite the presence of F3. In particular, ~40% of dn-N1-transfected cells were CNPase+, but these cells were less bifurcated (Fig. 12e, inset), compared to surrounding non-transfected cells, indicating a relatively immature stage. However, CNPase+ DTX-D2-transfected cells were hardly detectable. Consistent with a previous report (Wang et al., 1998), caN1-transfected cells remained Ng2+ and almost none became CNPase+ OLs (Fig. 12k), indicating that F3-induced NICD is specifically required for accelerated OPC differentiation. These results demonstrate that F3/Notch signaling via DTX1 promotes OPC development.

#### NB-3 is located at the paranode

Central to the inventor's aims is the characterization of an interaction between an axonal ligand and an oligodendroglial receptor at the paranode, acting as a stop signal to prevent extending oligodendrocyte cellular processes from impinging upon axonal domains destined to become nodes of Ranvier. The first step was to demonstrate the identity of the axonal ligands. Among the potential candidates, there is F3 which exists as a complex



with Caspr at the paranode (Rios et al, 2000). Previous results (Kazarinova-Noyes et al, 2001) are in agreement, showing that in rat optic nerve fibres, double-labelling with Caspr revealed the presence of F3 in both paranodal and nodal locations.

5

Recently, NB-3, a GPI-linked cell adhesion molecule, has been identified as a member of F3/Contactin family (Lee et al, 2000). To determine the cell type(s) that express NB-3, purified primary neurons, OLs and astrocytes from E17 rat cerebella were  
10 separately cultured and double stained for NB-3 and specific markers: neurofilament 200 (NF200) for neurons (Fig. 14Aa), galactocerebroside (Gal-C) for OLs (Fig. 14Ab) and glial fibrillary acidic protein (GFAP) for astrocytes (Fig. 14Ac). The results confirmed that only neurons expressed NB-3.  
15 NB-3 expression in rat brain stem development was next investigated by Western blot. Compared with F3, NB-3 expression started from E17, reached a plateau between P0 to P21, and declined afterwards (Fig. 14Ad), which parallels the time frame of OL development, as marked by increased expression of MAG. These observations  
20 indicate that NB-3 is a neuron-derived molecule. These results suggest that NB-3/Notch signalling may play certain roles in multiple phases of OL development. However, NB-3's exact localization and physiological role has not been fully clarified.

25 F3/contactin and TAG-1, two members of the F3/contactin family, locate specifically at nodal, paranodal, and juxtaparanodal regions (Girault and Peles, 2002). In particular, the paranode flanks the node of Ranvier and forms the adhesive site for axoglial junctions, which is crucial for initiating myelination  
30 and stabilization of myelin loops (Girault and Peles, 2002). To explore the role of NB-3 in axoglial interaction, NB-3 distribution was analysed along myelinated axons. Double immunofluorescence staining was performed by using monoclonal NB-3 antibody, which does not cross-react with F3 (not shown), and  
35 polyclonal antibodies against Caspr or sodium channels on adult

rat brain stem sagittal cryosections. Note that NB-3 overlapped exactly with Caspr (Fig. 14Ba-c) and flanked nodal sodium channels (Fig. 14Bd-f). To confirm this spatial specificity, NB-3 distribution in lipid rafts from P15 and adult (not shown) rat cerebral cortex was studied. F3 and Caspr are colocalized in lipid rafts. In agreement with the previous finding (Faivre-Sarrailh *et al*, 2000), these two paranodal molecules were colocalized in fraction 5 of a sucrose density gradient. Interestingly, NB-3 was also mainly located in this fraction (Fig. 14C). Taken together, these results indicate that NB-3 as a paranodal component may co-localize with the F3/Caspr complex on myelinated axons.

#### NB-3 is a functional ligand of Notch1

Given its expression profile and specific location during OL maturation, the inventor was interested in finding whether NB-3 could interact with OL-derived Notch1. Immunoprecipitation assay showed that Notch1 extracellular domain (~190 kDa) was detected in a NB-3 antibody-precipitate from adult rat brain membrane extracts while a Notch1 antibody-precipitate contained NB-3 (Fig. 15Aa), implying the existence of NB-3/Notch complex. OLN-93 (OLN) cells, a permanent cell line resembling maturing Ols (Richter-Landsberg and Heinrich, 1996) were used in cell adhesion assays. Immunocytochemistry showed that OLN cells expressed Notch1 on the surface (not shown). Cells adhered to the coated NB-3 substrate, but not to CHL1, another neural cell adhesion molecule (Holm *et al*, 1996). Adhesion was blocked by pre-incubation with NB-3 or Notch1 antibodies, but not with F3 antibody or pre-immune serum (Fig. 15Ab). The same results were obtained by using Notch1-transfected Hela cells (not shown). To map the binding site(s) on Notch1, four subcloned sequential equal-sized portions of the mouse Notch1 extracellular domain, labelled as N1.1, N1.2, N1.3, and N1.4 were used in a GST pull-down assay from rat brain lysates. Immunoblotting showed that NB-3 associated only with N1.3, a region containing EGF repeats 22-34 (Fig. 15Ac). The

specificity was confirmed by the observations that NB-3-transfected CHO cells plated upon the individual recombinant fragments bound predominantly to N1.3 (Fig. 15Ad) while mock-transfected CHO cells did not bind. Together, these results  
5 support the concept that NB-3 is a ligand of Notch1.

**NB-3/Notch interaction induces NICD nuclear translocation in OLN cells**

Upon binding its ligands, the core signalling mechanism of Notch  
10 involves Regulated Intramembrane Proteolysis (RIP) at the S3 site, which releases NICD into the nucleus (Schroeter et al, 1998). To explore whether NB-3 is a functional ligand of Notch1, OLN cells transfected with myc-tagged full-length mouse Notch1 (mNotch1-myc) were treated with NB-3 (Fig. 15Ba), Jagged1 (Fig.  
15 15Bb), or BSA (Fig. 15Bc) and immunostained with NICD antibody (Logeat et al, 1998). Both NB-3 and Jagged1, but not BSA, induced NICD to concentrate in the nucleus. Notch1 EGF antibody targeting EGF-like repeats on Notch1 prevented NB-3-induced NICD nuclear clustering (Fig. 15Bd). To study the properties of NB-3-induced  
20 NICD release, OLN cells were pre-incubated with  $\gamma$ -secretase inhibitor before exposure to NB-3 (Fig. 15Be) or Jagged1 (not shown). In both cases, nuclear NICD clustering was abolished, indicating that NB-3-induced NICD release involves  $\gamma$ -secretase. Moreover, OLN cells transfected with two S3 mutants: myc-tagged  
25 V1744K and V1744L which abolished S3 cleavage (Schroeter et al, 1998), successfully prevented NB-3- (Fig. 15Bf, g) as well as Jagged1- (Fig. 15Bh, i) induced NICD nuclear translocation. In Western blot (Fig. 15Bj), c-myc antibody precipitates from either NB-3 or Jagged1-treated V1744K-myc or V1744L-myc-transfected OLN  
30 cells could only be blotted by NICD antibody, which also recognizes intact Notch1 (~250 kDa) (upper panel), but not by V1744 antibody, which only recognizes released NICD from the S3 site. In contrast, mNotch-myc transfected OLN cells generated NICD (~120 kDa) that was blotted by V1744 antibody after NB-3 or  
35 Jagged1 treatment (lower panel). Together, these results

demonstrated that NB-3 induced  $\gamma$ -secretase-dependent Notch1 RIP at the S3 site.

***Hes1* and *Hes5* are not activated by NB-3**

5 In the nucleus NICD interacts with transcription factors, such as RBP-J and/or Deltex1 (DTX1), thus activating target genes, such as *HES* genes (Martinez Arias et al, 2002). Thus the correlation between Notch1 activation by NB-3 and endogenous *Hes1* mRNA level in OLN cells was investigated. Real-time PCR showed that compared  
10 to BSA, NB-3 induction resulted in the similar level of *Hes1* mRNA and oscillatory expression pattern (Ref) (Fig. 15Ca). 48 hours after various treatments, including another cell adhesion molecule L1, *Hes1* mRNA still remained at basal level (Fig. 15Cb). To further clarify whether NB-3 activates *Hes1*, Hes luciferase  
15 reporter assay (Fig. 15Cc) was used. Neither *Hes1* nor *Hes5* was activated by NB-3 treatment, while constitutive-active Notch1 (caN1) (Small et al, 2001) activated both luciferase reporters, indicating that NB-3 did not activate *Hes1* and *Hes5*.

**20 NB-3/Notch interaction upregulates MAG via DTX1**

The effect of NB-3/Notch1 signalling in myelination was further explored. The membrane extracts from co-cultured OLN cells with NB-3- or mock-transfected CHO cells were immunoblotted for MAG, a  
25 hallmark of OL maturation. MAG was upregulated only in OLN cells cultured with NB-3-transfected CHO cells (Fig. 16Aa). Real-time PCR showed that NB-3 increased MAG transcripts about 24 folds in primary OLs from P5-P7 rat cerebral cortex (Fig. 16Ab). Immunostaining showed that in response to NB-3, mNotch1-myc  
transfected OLN cells produced a 2.5-fold increase in MAG  
30 staining (Fig. 16Ac, f), compared with Jagged1 (Fig. 16Ad-f) or BSA (Fig. 16Af). MAG upregulation was blocked by pre-treating the cells with Notch1 EGF antibody (Fig. 16Af). To study the role of NB-3/Notch1 in MAG upregulation, OLN cells were transfected with dominant-negative Notch1 (dn-N1) (Small et al, 2001) that lacks  
35 the intracellular portion but still can bind to extracellular

ligands. After treatment with NB-3, MAG upregulation was not observed in transfected cells (Fig. 16Ba, green). Moreover, V1744K or V1744L-transfected OLN cells (green) also abolished MAG elevation (Fig. 16Bb, c). Since caN1 alone failed to increase MAG expression in transfected cells (Fig. 16Bd, e, green), these observations indicate that NB-3-generated NICD is essential for MAG upregulation.

#### NB-3-induced MAG upregulation involves DTX1

To identify the transcription factor involved in this event, OLN cells were transfected with dominant-negative RBP-J (dn-RBP-J) (Kato et al, 1997), which lack effective high affinity binding to DNA (Fig. 16Bh) or DTX1 (Yamamoto et al, 2001) (Fig. 16Bi) or two DTX1 deletion mutants that lack the Ring-H2 motif required for oligomerization of DTX1 (Fig. 16Bf, j, k): DTX1-D1 (Yamamoto et al, 2001) and DTX1-D2 (Izon et al, 2002). Hes1 luciferase reporter assay confirmed the validity of these constructs in OLN cells in that dn-RBP-J inhibited Hes1 transactivation by caN1 and the competitive binding of DTX1 to caN1 also affected this, which was restored by DTX1-D1 or DTX1-D2 (Fig. 16Bg). After NB-3 treatment, cells were immunostained for MAG and fluorescence intensity counted (Fig. 16B1). It was found that the ablation of Hes1 basal expression in OLN cells (green) by dn-RBP-J (Fig. 16Bh) did not affect NB-3-induced MAG upregulation. Similarly, DTX1-transfected cells (Fig. 16Bi, green) responded to NB-3 stimulation. However, the dysfunction of endogenous DTX1 by DTX1-D1 and DTX1-D2 (Fig. 16Bj, k, green) abolished this event, indicating that DTX1 is involved in NB-3-mediated MAG expression. Thus NB-3 may activate the Notch1 receptor and release NICD, which recruits DTX1 during myelination.

#### NB-3 developmentally clustering at paranodes promotes OPC differentiation via Notch1/DTX1 signaling pathway

Since NB-3 is expressed from E17, it is conceivable that the two pairs of interaction: Jagged1/Notch1 and NB-3/Notch1 may coexist

along the axon at early developmental stage. To explore whether NB-3 and Jagged1 coordinate myelination in a step-wise fashion, the spatial correlation between these two molecules along axons during development was first studied. Sections of P2, P5, and P14 (not shown) rat brain stems were double labelled for Jagged1 (green) and NB-3 (red). At P2, Jagged1 was evenly distributed along the axon and congregated NB-3 was hardly detectable (Fig. 17Aa), which was in agreement with the previous observation that Jagged1/Notch1 signalling may predominate to promote migration of young OL loops along axons at this stage (Wang et al, 1998). At P5, NB-3 clustered at the paranode (Fig. 17Ab, c) and Jagged1 occupied the juxtaparanode and internode, separated from paranodal NB-3 (Fig. 17A). Given the decrease of Jagged1 expression (Wang et al, 1998) after P6, the inventor's observations suggest that the balance between these two pairs of interactions may be disrupted during the later stage of OL maturation, in other words, NB-3/Notch1 interaction predominates to induce OL maturation at the CNS paranode. To attest this notion, purified OPCs from P7 Wistar rat optic nerve (Fig. 17Ba) were treated with BSA, NB-3 or Jagged1 for 2 days and then immunostained for Ng2 (red), a progenitor marker and CNPase (green), a young OL marker (Fig. 17Bb-d). Statistical counting showed that NB-3 promoted OPC differentiation into CNPase-positive OLs (~75%), compared to BSA (~50%) or Jagged1 (~0%) treatment (Fig. 17Bh). To further confirm the involvement of Notch1 and DTX1 in NB-3-promoted OPC differentiation, OPCs were transfected with dn-N1 (Fig. 17Be) or DTX1-D1 (Fig. 17Bf) and treated simultaneously with NB-3. Double labelling for tags (green) and CNPase (red) showed that introduction of either construct significantly blocked NB-3-promoted OPC differentiation into OLs (Fig. 17Bh). And consistent with the previous study (Wang et al, 1998), caN1-transfected OPCs remained undifferentiated (Fig. 17Bg, h).

**NB-3/Notch signalling via Deltex1 directs differentiation of embryonic neural stem cells into oligodendrocytes**

To explore whether NB-3/Notch1 interaction is also involved in oligodendroglialogenesis from Neural Stem Cells (NSCs), the developmental expression patterns of NB-3 and Notch1 was investigated. Western blot of rat brain samples showed that both NB-3 and Notch1 were expressed at embryonic day 17 (E17) and an abrupt increase after birth reached a maximum level between postnatal day 0 (P0) and 21 (P21), which corresponds to the time frame of oligodendroglialogenesis from NSCs (Fig. 18).

**NSCs: NB-3 is a functional ligand of Notch1**

The expression of Notch1 on NSCs by immunofluorescence was studied next. The NSCs used in this study were isolated from embryonic day 14 BALB/c mouse embryo striatum (Arsenijevic et al, 2001). These cells expressed intermediate filament protein nestin (Fig. 19Aa), a progenitor marker, and Notch1 (Fig. 19Ab, c). To confirm the NB-3/Notch interaction previously observed, NB-3-Fc fusion protein coupled to Protein A beads was used to precipitate binding partner of NB-3 from NSC membrane extracts. The precipitate was positively blotted with Notch1 antibody (Fig. 19Ba). Further, P0 rat brain membrane extracts were immunoprecipitated using NB-3 antibody and blotted with Notch1 antibody and vice versa (Fig. 19Ba). Western blot showed that NB-3 and Notch1 co-immunoprecipitated. Moreover, cell adhesion assay was performed by plating murine Notch1- (N1) and mock-transfected HeLa cells onto NB-3 substrate coated proteins. N1-transfected HeLa cells adhered to NB-3 (Fig. 19Bb), but mock-transfected HeLa cells did not bind (Fig. 19Bf). Adhesion was prevented by pre-incubation of cells with NB-3 or Notch1 antibodies (Fig. 19Bc, d), but not with pre-immune serum (Fig. 19Be). These studies corroborate that NB-3 is a binding partner of Notch.

NSCs: NB-3 induces NICD nuclear translocation but does not activate *Hes1*

Notch activation by ligand binding is featured by NICD nuclear translocation (Schroeter et al, 1998). The inventor studied whether NICD nuclear translocation in NSCs could occur in response to NB-3 stimulation. It has been observed above that NB-3 induces  $\gamma$ -secretase dependent NICD nuclear translocation in OLN cells. In agreement with that study, merged triple staining showed that NB-3 treatment of nestin (green) positive NSCs resulted in NICD clustering (red) in the nucleus visualized by Hoechst 33258 staining (blue) (Fig. 19Ca), which was similar to Jagged1 stimulation (Fig. 19Cb). However, BSA failed to trigger this event (Fig. 19Cc), suggesting that NB-3 specifically interacts with Notch1 to effect typical NICD nuclear translocation. Another prominent feature of classic Notch signalling pathway is that nuclear NICD transactivates target genes, such as *Hes* genes (Martinez Arias et al, 2002). Thus the *Hes1* luciferase reporter assay was utilized to investigate whether NB-3-generated NICD could activate *Hes1*. The results showed that NB-3 did not upregulate *Hes1*, while either Jagged1 or constitutive-active Notch1 (caN1) (Small et al, 2001) activated *Hes1* as expected (Fig. 19Da). As increasing evidence showed that Deltex1 (DTX1) is another Notch downstream element (Yamamoto et al, 2001), the impact of NB-3 on DTX1-mediated Mash1 transcriptional activity in pE7 luciferase reporter assays was studied (Fig. 19Db). Consistent with the previous report (Yamamoto et al, 2001), DTX1 inhibited Mash1 transactivation of pE7 reporter. However, NB-3 treatment mimicked this inhibition partly, which was abolished by one DTX1 deletion mutant, Flagged-tagged DTX1-D2 (Izon et al, 2002) that lacks the domain 3, Ring-H2 motif which is required for functional DTX1 homodimer formation (Matsuno et al, 2002) (Fig. 21c). These results suggest that NB-3-generated NICD may mediate DTX1-related transcription events.

35



**NSCs: NB-3 promotes OL generation**

Given that the expression of both NB-3 and Notch1 parallel the time frame of OL development, the inventor explored whether NB-3/Notch interaction was involved in oligodendroglioneogenesis from NSCs. To do this, NSCs were allowed to differentiate for 7 days in the absence of mitogen and in the presence of serum and NB-3 (Fig. 20a, d), BSA (Fig. 20b, e), or Jagged1 (Fig. 20c, f). The cells, identified by Hoechst 33258 (Fig. 20a-f, blue), were immunostained for OL marker: CNPase (Fig. 20a-c, red); neuronal marker:  $\beta$ -tubulin (Fig. 20d-f, red); and astrocyte marker: GFAP (Fig. 20a-f, green). The results showed that distinct from BSA and Jagged1, NB-3 promoted OL generation. On the other hand, compared to BSA, NB-3 had little impact on neurogenesis, while Jagged1 inhibited this as reported (Morrison *et al*, 2000). Flow cytometry confirmed these observations in that NB-3 induced a 2-fold increase in OL generated, compared to BSA treatment, while Jagged1 inhibited OL development (Fig. 20g).

**NSCs: NB-3/Notch signalling pathway via DTX1 instructs oligodendroglioneogenesis**

To confirm the involvement of Notch1 in this event, NSCs were transfected with V5-tagged dominant-negative Notch1 (dn-N1) (Small *et al*, 2001). dn-N1 lacks the intracellular domain but can still bind to the extracellular ligand. The Hes1 luciferase reporter assay showed that dn-N1 failed to respond to Jagged1 treatment to activate Hes1 (Fig. 21h). Mitogens were then withdrawn and NB-3 added to the culture medium. Double labeling for V5 and CNPase or GFAP showed that dysfunction of Notch1 by dn-N1 abolished NB-3-promoted oligodendroglioneogenesis, while favouring astrocyte formation (Fig. 21a, b, i). To further investigate whether DTX1 participates in NB-3-induced OL formation, NSCs were transfected with Flag-tagged DTX1-D2 (Fig. 21c). Hes1 luciferase reporter assay confirmed that DTX1 inhibited Hes1 activation by caN1 while DTX1-D2 restored Hes1 elevation by caN1 (Fig. 21h). After differentiation in the presence of NB-3, NSCs were immunostained

for Flag and CNPase or GFAP. The results showed that DTX1-D2 transfected cells failed to differentiate into OL after NB-3 stimulation but were directed to astrocytes (Fig. 21d, e, i). And consistent with previous studies (Tanigaki et al, 2001)

5 introduction of caN1 into NSCs resulted in astrogliogenesis while inhibiting OL generation (Fig. 21f, g, i). These observations indicate that NB-3 promoted oligodendroglialogenesis via Notch/DTX1 signalling pathway (Fig. 22).

10 Discussion relating to the Second Aspect of the Invention

Using molecular, cellular, and morphological approaches, the inventor has identified a functional molecular interaction between Notch and F3/NB-3 at the paranode, a vital site for axoglial signalling in myelination. A new axonal adhesion  
15 molecule - NB-3, has been localized to the paranode. This interaction may regulate the differentiation of the myelinating oligodendrocyte during axonal ensheathment and act to prevent putative nodes of Ranvier from becoming invested in myelin. In addition to established Notch/Jagged1 signaling pathway, the  
20 inventor provides evidence for novel Notch ligands - F3 and NB-3 in the context of the myelinating process, suggesting that signaling via Jagged1 and F3/NB-3 may contribute in a co-ordinated fashion to myelination.

25 The data designates the adhesion molecule F3 as a new functional ligand of Notch. F3/Notch interaction induces the generation and nuclear translocation of NICD and elevates Notch1 and Notch2 expression. It promotes OPC differentiation and upregulates MAG in OLN cells and primary OLs, thus revealing a potential  
30 regulatory role for F3 in OL maturation. Thus, ligand-specific Notch signaling via Jagged1 as an inhibitory factor and F3 as a positive instructor may regulate myelination in a coordinated fashion (Fig. 13).

The data also designates NB-3 as another new functional ligand of Notch. The extracellular NB-3/Notch interaction releases from the membrane NICD, which recruits DTX1 and translocates into the nucleus where the complex mediates directly or indirectly CNPase expression, thus promoting oligodendroglialogenesis (Fig. 22).

**Notch is an oligodendroglial surface receptor of F3/NB-3**

Intuitively, both axons and oligodendrocytes must actively participate and achieve two-way communication if myelination is to be properly co-ordinated. The emerging concept is that of an active signaling axoglial channel sited at paranodes, where cytoplasm-filled glial loops spiral and indent the adjacent axolemmal membrane. Although paranodal axonal and glial molecular members have been described, their functional interactions in regulating myelination remain ill-defined. In support of the notion that glial Notch could act as the receptor for axonal F3 and NB-3, the inventor shows through co-immunoprecipitation experiments and GST pull-down assays that these molecules physically interact. Furthermore, he has identified two regions of F3 interaction (N1.1 and N1.3) in the extracellular domain of Notch1, one of which (N1.3) is shared with or overlaps with that involved in NB-3 binding.

**Notch is a functional receptor for F3/NB-3**

This study proposes the presence of a molecular stop signal to prevent mature myelinating oligodendrocytes from encroaching upon and myelinating putative nodes of Ranvier. The inventor has direct evidence of oligodendroglial processes emanating from OLN-93 cells terminating and spreading over the surface of F3 transfected CHO cells. The molecular basis for this signal is an interaction between F3 and Notch. In support of this, adhesion assays using OLN-93 and Notch1-transfected HeLa cells cultured upon a F3 substrate demonstrate that cellular interactions are disrupted by specific antibodies against the proposed ligand-receptor pair. Together with previous morphological data that

confirm the presence of F3 at the paranodes and the inventor's data that reveal surface expression of Notch receptors on oligodendrocytes, the inventor suggests that an F3 signalling interaction indeed exists at the paranode and acts to prevent further extension of oligodendroglial processes. This interesting outcome is unique in postulating a mechanism that regulates the lateral limits of the myelinated internodes. In a similar manner to F3, the inventor has also provided evidence that NB-3 is a ligand for Notch1. Plating the Notch1-expressing HeLa cells on F3 or NB-3 led to different interactive outcomes; adherence to the NB-3 substrate, but repulsion from the F3 substrate. This could be due to distinct signals originating from the dual site F3-Notch interaction (resulting in cell repulsion) and the single site NB-3-Notch interaction (mediating adhesion). OLN-93 cells plated on NB-3 undergo a rapid differentiation and morphological alteration which ultimately leads to a flattened sheet-like appearance of the cell body, possibly representing a differentiation step in the multi-phase process of myelination as the oligodendrocytes prepare to ensheath contacting axons. However, the common theme underlying the F3/ Notch and NB-3/ Notch interactions is that both are linked to myelination as evidenced by the upregulation of MAG at both protein and mRNA levels.

**25 Different ligands signal to glial-derived Notch to co-ordinate myelination**

The complex nature of myelination demands a co-ordinated, dynamic series of axon-glial interactions which are likely regulated by the temporal and spatial distribution of *trans* interacting components. One such regulated interaction involves the interaction of axonal Jagged1 and oligodendrocyte-derived Notch1 in rat optic nerve (Wang et al, 1998). Notch receptor activation by Jagged1 inhibits differentiation and promotes migration of oligodendrocyte cellular processes along the axon. In an optic nerve model, Jagged1 is downregulated with a time course that

parallels myelination. In accord with the finding that myelination begins only after target innervation (Schwab and Schnell, 1989), further studies showed that Jagged1 downregulation occurred only after axons reached their targets (Dugas et al, 2001). The Jagged1/ Notch signal that keeps oligodendrocytes in an immature state thus abates and differentiation of oligodendrocytes into mature myelin-forming cells progressively occurs. This key signal may then allow contact and extension of oligodendroglial cellular processes along the axon. The inventor's results support the idea that the Notch receptors may switch to other ligands, such as clustered F3/NB-3 as positive signals at paranodes, triggering the onset of the ensheathing process. This ligand switch mechanism may also underlie a phase transition from differentiating oligodendrocytes held in check by an axonal inhibitory signal to subsequent maturation into myelinating cells.

Spatial regulation of myelination may be achieved by the clustering of molecules along the axon. Clustering of sodium channels has been described (Salzer, 1997). In the maturing nerve, Caspr molecules are progressively herded towards the node to form tight bands, the "Caspr spiral", on either side of it (Pedraza et al, 2001).

Oligodendrocytes themselves appear crucial in promoting paranode formation through directing molecular localization. FIAU-induced ablation of oligodendrocytes in MBP-TK led to mis-localization of Caspr (Mathis et al, 2001). Significantly, the few myelinating oligodendrocytes in the MBP-TK brains were associated with focal clusters of Caspr close to MBP-positive domains. In *jimpy* mice, spontaneous degeneration of oligodendrocytes and mis-localization of Caspr occurs (Mathis et al, 2001). The inventor's previous work has also shown in re-myelinating peripheral nerves how F3, initially diffusely distributed along the demyelinated nerve fibre, becomes clustered at the paranodes by the effect of re-

myelinating Schwann cells. Specifically, F3 clustering is seen at the extending edge of the Schwann cell (Kazarinova-Noyes *et al*, 2001). The results provided herein suggest that NB-3 also undergoes a similar clustering process, as it is barely  
5 detectable along P2 axons but forms distinct pairs of bands corresponding to paranodal staining at P5 and P14 (Fig. 4). This result also supports the notion that oligodendrocytes may be required for clustering of axonal molecules, such as Caspr, F3, and NB-3.

10 The oligodendroglial-dependent clustering may not only result in a redistribution of axonal proteins into domains but also lead to a "dose effect", in that molecules that were once sparsely distributed become packed together in highly dense bands. Only  
15 the concentrated axonal ligand may productively signal to glial receptors to achieve the "stop" effect, explaining why stop signals are not prematurely activated at the initial phase of axoglial contact when the axonal molecules are diffusely distributed. In this way, clustering could promote the switch of  
20 axonal cues, for example for Notch receptor switching from internodal Jagged1 to paranodal F3 and NB-3. This switch in cues would represent an essential regulatory mechanism during axonal ensheathment. In essence, the myelinating oligodendrocyte can be likened, albeit simplistically, to a machine with excavators at  
25 its lateral tips, its motor engine started (oligodendrocyte differentiation triggered by downregulation of Jagged1/Notch inhibitory signal), heaping up larger and larger mounds of earth as it progresses forwards (clustering of nodal and paranodal molecules) and then braking to a halt via an in-built feedback  
30 mechanism (clustered "high dose" F3 signalling to oligodendroglial Notch).

A number of studies have addressed signalling mechanisms that may play a part during myelination, such as the roles of neurofascin  
35 (Martin-Collinson *et al*, 1998) and N-cadherin (Schnadelbach *et*

al, 2001). The inventor favours the concept proposed by Pedraza  
et al (2001) that membrane proteins of the axoglial junction at  
paranodes, as a conduit for bi-directional signal transduction,  
may act as selective molecular sieves and diffusion barriers  
5 whose purpose is to contribute to the organization of axonal  
domain architecture. Membrane proteins of the apposing axolemma  
and glial membrane loop interact via adhesion molecules to form  
mobile constructs of molecular sieves and barriers that "travel"  
along the axolemmal surface, pushing sodium channels and packing  
10 them at the nodes and allowing potassium channels to pass through  
to reach the juxtaparanode. Intracellular proteins linked to the  
cytoplasmic domains of the axolemmal and glial molecules may also  
contribute in a significant fashion to this barrier/sieving  
effect. Given that both F3 and NB-3 are GPI-linked molecules on  
15 the axonal surface, it will be interesting to investigate their  
cis interactions with transmembrane proteins and the nature of  
associated intracellular signal transduction pathways. Caspr,  
which associates with F3, interacts with members of the protein  
4.1 family (Baumgartner et al, 1996; Ward et al, 1998) via its  
20 intracellular segment. This connects Caspr to spectrin and the  
axonal cytoskeleton (Hoover and Bryant, 2000). The presence of  
intact nodes of Ranvier containing clustered sodium channels but  
abnormally distributed F3, Caspr and potassium channels in the  
CGT-deficient mouse testifies to the fact that other important  
25 signalling mechanisms may exist (Coetzee et al, 1996; Bosio et  
al, 1998). As this enzyme (UDP-galactose-ceramide  
galactosyltransferase) is required for galactolipid synthesis,  
lipid molecules may also be crucial regulators of myelination.  
Further work to define the configuration and components of the  
30 axoglial junction will no doubt help create a knowledge base from  
which it is hoped that meaningful strategies to promote re-  
myelination of the damaged CNS will arise.

This study also reveals a new facet of Notch signalling - the  
35 existence of F3 and NB-3 as signalling ligands. These neural cell

adhesion molecules are relatively widely distributed in the nervous system and may regulate Notch mediated processes. At present, Notch receptors are mostly investigated in the context of DSL (Delta, Serrate, Lag-2) ligands, CSL (CBF1, Suppressor of hairless, Lag-1) transcriptional cofactors and gene targets which are categorized as the HES (Hairy/ Enhancer of Split) family of basic helix-loop-helix transcriptional regulators. The Notch receptor classically plays a critical role in cell fate selection during development (Artavanis-Tsakanos et al, 1999; Baker, 2000; Mumm and Kopan, 2000). Apart from neurogenesis during development, Notch also participates in gliogenesis (Morrison et al, 2000) and has been implicated in T-lymphocyte development (Robey et al, 1996), haematological malignancies (Ellisen et al, 1991) and familial stroke syndromes (Joutel et al, 1997). Notch is also unique as one of a few proteins that undergo regulated intramembrane proteolysis (Weinmaster, 2000). The modulation of these processes by cell adhesion molecules thus becomes another avenue for research.

**Notch is an oligodendroglial surface receptor of F3**

Adhesive contacts at axoglial junctions are partly contributed by the F3-Caspr-neurofascin 155 heterotrimer (Girault and Peles, 2002), but the exact role of this complex remains to be further characterized. Herein the inventor defines a novel ligand-receptor interaction between F3 and Notch. Cell adhesion/repulsion assays and biochemical approaches demonstrate that Notch and F3 are binding partners. Further two extracellular sites on Notch1 for F3 binding have been identified, namely, N1.1 and N1.3. The former contains the EGF repeats 11-12 involved in DSL binding (Rebay et al, 1991). F3 is also expressed on OLs (Koch et al, 1997) and transduces signals to glial intracellular Fyn which then interacts with Tau protein to mediate myelination (Klein et al, 2002). Since soluble F3 is sufficient to trigger F3/Notch signaling and the lipid raft assay demonstrated that Notch1 is not localized to the F3-enriched fraction, F3 may not



interact with Notch1 in *cis*. Given that Notch and F3 co-localize in various regions of the brain (Lardelli et al, 1994; Revest et al, 1999), particularly at axoglial junctions, the results suggest a role for F3 as a *trans*-acting ligand of Notch.

5

**F3/Notch signaling induces proteolytic release of NICD and upregulates Notch homologs**

RIP, the generation of nuclear signaling proteins derived from non-nuclear precursors such as Notch and APP, is a new paradigm of signal transduction that potentially adds unforeseen diversity to the signaling repertoire of a cell (Ebinu and Yankner, 2002). As with DSL, the inventor has shown that F3 binding triggers Notch intramembrane proteolysis at the S3 site and nuclear translocation of the resultant NICD in OLN cells. The ability of Notch1 EGF (EGF 11-12) antibody, or brefeldin A and monensin, to block this event suggests that the extracellular F3/Notch interaction is essential for the intramembrane cleavage-derived generation and transport of NICD. Thus, it is another example of RIP. In addition, the F3/Notch signaling pathway may activate a feedback loop that specifically increases Notch1 and Notch2 (but not Notch3) expression or protects Notch1 and Notch2 from rapid degradation. Either way, this may serve to replenish consumed receptors on the cell surface and thus sustain signal continuity.

**25 A possible model for F3/Notch signaling via DTX1**

Given that F3 and Jagged1 share a common binding domain on Notch1, Jagged1 downregulation that occurs prior to myelination may act to permit the alternate interaction of Notch with F3. In OLN cells, both Jagged1 and F3 trigger  $\gamma$ -secretase-mediated and S3-directed release of NICD followed by its nuclear translocation. Moreover, caN1 transactivates *Hes1*, which is blocked by co-expression of dn-RBP-J-myc or DTX1. Thus it is conceivable that F3-induced Notch intracellular signaling is associated with RBP-J. However, only F3-induced NICD, but not

Jagged1-induced NICD or *can1* and *can2*, increases MAG expression, suggesting that this event may require specific extracellular ligand-receptor interaction. Moreover, experiments utilizing either dn-RBP-J-myc or *Hes1* antisense oligonucleotides indicate  
5 that the blockage of endogenous *Hes1* expression is not involved in F3/Notch-induced MAG upregulation. On the other hand, two truncated mutants of DTX1, which lack the Ring-H2 finger motif that is indispensable for the formation of functional homodimeric DTX1 (Matsuno et al, 2002), prevent both F3-promoted OPC  
10 differentiation and MAG upregulation. Thus it is proposed that a switch in ligands may alter Notch intracellular signaling effectors (Fig. 13). The binding of different ligands may induce the formation of distinct Notch conformations. Such conformational alterations could result from different, albeit  
15 overlapping, regions of Notch recognized by Jagged1 and F3. Notch receptors with distinct conformations may interact, before or after cleavage, with different cytoplasmic factors, such as DTX1. The form of NICD subsequently arriving at the nucleus then specifies further potential interactions and determines its  
20 transcriptional target. It will be of significance to investigate this hypothesis and identify DTX1-related transcription cofactors that are required for F3/Notch signaling.

#### Potential role of F3/Notch signaling via DTX1 in OL maturation

25 Jagged1/Notch signaling contributes to maintaining OPCs in an undifferentiated stage (Wang et al, 1998). The failure of efficient remyelination in multiple sclerosis (MS) has been partly attributed to the activation of OPC Notch by astrocyte-expressed Jagged1 (John et al, 2002). However, Jagged1 expression  
30 sharply decreases from around P6 (Wang et al, 1998), a time point concurrent with the onset of myelination and the clustering of axonal F3 at the paranode (Kazarinova-Noyes et al, 2001), an ideal position to interact with Notch receptors on the surface of newly formed OLs. Mutant animals, in which Notch1 is selectively  
35 ablated in OPCs (Genoud et al, 2002), are characterized by

ectopic immature OLs, most of which undergo apoptosis, indicating that autonomous differentiation in the absence of the Notch receptor may be disruptive and that other regulatory signaling cascades besides Jagged1/Notch may be needed to ensure correct differentiation and survival of OLs. It was observed here that F3 promotes OPC differentiation, which can be blocked by both dominant-negative Notch and DTX1 deletion mutants, and oligodendroglial processes emanating from OLN cells terminate and spread over the surface of F3-transfected CHO cells, an event related to the upregulation of myelin-specific proteins, such as MAG and CNPase. Upon Notch interaction with either immobilized or soluble F3, MAG, a specific marker of mature OLs, is significantly upregulated at both the protein and mRNA levels. MAG upregulation can be blocked by dominant-negative construct of Notch1, Notch2, or deletion mutants of DTX1. These observations indicate that OL maturation involves F3/Notch signaling via DTX1 (Fig. 13).

In summary, the study reveals a new facet of the Notch signaling pathway. Upon activation by F3, Notch signaling via DTX1 continues to participate in OL maturation through upregulating certain myelin-related proteins instead of solely functioning to inhibit OPC differentiation into OLs. Hence, this finding may prove to be an efficient molecular handle for promoting remyelination in degenerative diseases, such as MS, by creating an environment in which Notch predominantly interacts with endogenous or exogenous F3 to initiate the F3/Notch/DTX1 signaling pathway.

**NB-3 is a new constituent of the paranodal architecture**  
The construction of a myelinated axon during nervous system development is a well-orchestrated phenomenon, yet the molecular details of this process remain unclear. Apart from the segmental nature of myelin ensheathment, a redistribution of axonal molecules occurs to create distinct axonal domains in the mature

nerve fibre. The molecular architecture of the various axonal domains has been extensively reviewed (Morell and Quarles, 1999; Arroyo et al, 2000; Peles and Salzer, 2000; Denisenko-Nehrbass et al, 2002). Constituents of the paranode include axonal F3 in association with the neurexin superfamily molecule, Caspr, and oligodendroglial neurofascin-155 (NF-155) (Rios et al, 2000; Tait et al, 2000). Studies of F3-null mice highlight F3 as a key player in the synthesis of the axoglial apparatus in the peripheral nervous system (PNS). Prior to their death by postnatal day 18, the mutant mice have an ataxic phenotype with hindlimb weakness, attributed to defective connections involving cerebellar interneurons and to reduced nerve conduction velocity and excitability (Berglund et al, 1999; Boyle et al, 2001). The latter problem with nerve impulse conduction reflects abnormalities in axoglial junction formation in myelinated peripheral nerve. Consistent with a paranodal function of F3, these abnormalities include disruption of the axoglial junction and defective transport of Caspr, a *cis* binding partner of F3, to the axon membrane (Boyle et al, 2001). The fact that adhesive axoglial interactions still form in the F3 mutant mice points to the involvement of other paranodal molecules, with NB-3 being one such candidate. Although the distribution/localization of NB-3 in the PNS is unknown, in the CNS NB-3 is expressed in the olfactory bulb, layers I, III and V of the cerebral cortex, piriform cortex, anterior thalamic nuclei, locus coeruleus, mesencephalic trigeminal nucleus and the Purkinje cells of the cerebellum (Lee et al, 2000). The inventor shows that NB-3 clusters and co-localizes with Caspr at paranodes from P5 to adulthood, certainly suggesting the likelihood of it being similarly localized in the PNS.

NB-3 clustering at the CNS paranode promotes maturation of oligodendrocyte precursor cells via Notch/Deltex1 signalling pathway

In summary, the above study has shown that NB-3, a neuron-derived molecule, is a functional ligand of Notch1. Acting as a spatial switch signal by developmentally clustering at the CNS paranode, it releases NICD at the S3 site via RIP and triggers Notch/DTX1 signalling pathway, which promotes OPC differentiation and OL maturation to coordinate myelination.

**NB-3/Notch signalling via Deltex1 directs differentiation of embryonic neural stem cells into oligodendrocytes**

Multiple sclerosis (MS) is a chronic demyelinating disease in the CNS. Neural stem cell transplantation is a promising treatment for such diseases (Pluchino *et al*, 2003). It will be of significance to establish a reliable method for selectively predifferentiating appropriate donor stem cells to OPCs or OLs. In the present study, pre-clinical validation has been provided of a novel signalling pathway allowing for the first time using NB-3, a neuronal cell recognition molecule, to direct neuronal stem cells to OPC and/or OLs lineages *in vitro*. Moreover, recent studies have shown that the failure of efficient remyelination in MS is partly attributed to the activation of OPC Notch receptor by astrocyte-expressed Jagged1 (John *et al*, 2002). The ectopic immature OLs and subsequent apoptosis appear in mutant mice in which Notch1 signalling is selectively inhibited in OPCs (Genoud *et al*, 2002). These observations implicate that, in addition to Jagged1/Notch1 interaction, there may exist other axon-derived Notch1 ligands that mediate OL differentiation by properly activating the Notch1 signalling pathway (Hu *et al*, 2003). In support of this notion, the inventor's present observations in NSCs incriminate that NB-3/Notch signalling via Deltex1 may modulate OL differentiation from NSCs and thus represent a potential target for therapeutic intervention in demyelinating diseases.

**Materials and Methods Relating to the Second Aspect of the Invention**

**Antibodies**

Monoclonal Notch1 EGF repeats 11-12 (Neomarker), CNPase, Flag, beta-tubulin and MAP2 (2a+2b) (Sigma), c-myc (9E10) and HA (Santa Cruz Biotechnology), nuclear matrix protein, Gal-C and nestin (Chemicon), GFAP (DAKO) and V5 epitope (Invitrogen) antibodies; 5 polyclonal V1744-cleaved NICD (Cell Signaling), N2ICD, Jagged1, Jagged2, Delta (Santa Cruz Biotechnology),  $\gamma$ -tubulin (Sigma), Ng2, sodium channels (NaCh), NF200 (Chemicon), Hes1 (Kaneta et al, 2000), NICD (Logeat et al, 1998), Notch1, Notch2, Notch3 10 (kind gifts from Dr. Lendahl) and F3 (Shimazaki et al, 1998).

Polyclonal Caspr antibody was obtained by immunization of rabbits with a GST fusion protein of amino acids 277-430 of human Caspr 15 and polyclonal MAG antibody (7610) by immunization of rabbits with the following peptide: N'-CISCGAPDKYESREVST-C' (Eurogentec).

Secondary antibodies conjugated to Cy2, Cy3 and FITC were obtained from Amersham Pharmacia Biotech. Inc. 20

Anti-NB-3 serum and monoclonal antibodies were generated against recombinant protein expressed in *E. coli* transformed with the pET15b vector (Novagen) containing rat NB-3 cDNA encoding Ig domains I-II (amino acids 30-227). To construct this expression 25 vector, two oligonucleotide primers were used in PCR reactions to amplify cDNA encoding the corresponding region from a cDNA library synthesized from rat brain total RNA. The two oligonucleotide primers are:

5'-TCCGGATCCCATGGAGCCACAGGATGTCATTTT-3' (forward)  
30 5'-TCCGGATCCGTCGACTGGCACATATTCCCCCATGA-3' (reverse),

The PCR was carried out for 30 cycles at 94 °C for 30s, 60 °C for 30s and 72 °C for 45s after denaturation at 94 °C for 3 min. The amplified cDNA fragments were digested with BamHI and inserted

into a BamHI-cut pET15b vector after DNA sequencing. The protein was expressed in *E. coli* BL21(DE3) pLysS by induction with IPTG. It was partially purified as inclusion bodies, and these were solubilized and applied to SDS-PAGE and the recombinant protein  
5 eluted electrophoretically from the gel. The protein was used to immunize rabbits for antiserum and BALB/c mice for monoclonal antibodies (Harlow, 1998).

#### Cell co-culture

10 F3 transfected CHO cells (Gennarini *et al*, 1991), TAX, TAG-1 and mock transfected CHO cells (Furley *et al*, 1990; Tsiotra *et al*, 1993) were co-cultured with the oligodendrocyte cell line OLN-93 (Richter-Landsberg and Heinrich, 1996) in a 2:1 ratio in Dulbecco's modified Eagle's medium (DMEM, Gibco), 10% fetal calf  
15 serum (Gibco) and penicillin/streptomycin (Gibco) for 2 days at 37°C in a humidified atmosphere. Cells were pre-stained with the PKH26 red fluorescent cell linker kit (Sigma). The cells were also stained with primary monoclonal antibodies to c-myc (Santa Cruz Biotechnology) and Cy-2 labelled goat anti-mouse secondary  
20 antibodies (Sigma). To determine the number of the stopped processes in the co-cultures, OLN-93 cells (more than 100 cells) from three randomly selected areas in each of three cover slips were counted per experiment. Raw data from at least three independent experiments were analyzed by analysis of variance and  
25 then Newman-Keuls test with  $p < 0.05$  and  $p < 0.01$  being considered significant or highly significant, respectively.

NB-3- or mock-transfected CHO cells were cultured with OLN in a 2:1 ratio for 2 days. The membrane portions of the co-cultures  
30 were extracted as described (Xiao *et al*, 1996).

**Transfection and Characterization of NB-3-transfected CHO cells**  
CHO cells were transfected with 10 µg of pcDNA3-NB-3 using lipofectin (GIBCO BRL) according to the manufacturer's  
35 instructions. Following drug selection with G418 (Life

Technologies, Inc), surviving cell clones were expanded and analyzed by Western blot and immunohistochemical staining for surface NB-3 expression.

5    **Transfection of Cells**

OLN cells were transiently (V1744K-myc, V1744L-myc, dn-N1-V5, dn-N2-V5, pcDNA4/V5/LacZ (Invitrogen), dn-RBP-J-myc, DTX1-myc, DTX1-D1-HA, DTX1-D2-Flag, caN1, caN2) or stably (mNotch1-myc) transfected using Lipofectamine (Invitrogen). CHO cells were  
10    stably transfected with full-length Jagged1 (Small et al, 2001) using Lipofectamine. The stable transfectants were screened with 400 µg/ml G418 (Sigma) or 250 µg/ml Zeocin (Invitrogen) and identified by immunostaining and Western blot.

15    **Immunocytochemistry**

Cells were cultured on 13mm coverslips (Nalge Nunc International). After various treatments, including γ-secretase inhibitor (Sigma), cells were fixed with 4% paraformaldehyde and blocked with 1% BSA. Cells were then incubated with primary  
20    antibodies in 0.2% BSA for 1 hour, followed by Cy3-labeled or Cy2-labeled secondary antibody (Sigma). After mounting in fluorescent mounting medium (DAKO), cells were visualized with a Leica DM RXA2 fluorescent microscope. The photos were taken using the same optical parameters to ensure the comparable luminosity.  
25    At least ten different viewing fields from three independent experiments were used to calculate the percentage of cells showing NICD translocation or differentiation. Two hundred cells from at least three independent experiments were quantified for fluorescence intensities by Adobe Photoshop™ (Jack et al, 2001)  
30    and measured for cytoplasmic area by Leica QFluoro software. The raw data were analyzed by Student's t test with p<0.05 and p<0.01 being considered a significant or highly significant difference, respectively.



- For detection of primary NF200, GFAP and Gal-C antibodies, Alexa Fluor 488-conjugated anti-mouse IgG or Alexa Fluor 546-conjugated anti-rabbit IgG (1:1500; Molecular Probes) were used. For transfected NSCs, cells were first transfected with plasmid caN1, DTX-D2, dnN1 using Lipofectamine 2000 (Invitrogen) and treated with or without NB-3 in serum free culture medium without growth factor for 24 hours, and then cells were allowed to differentiate on 13 mm coverslips in 1% FCS medium.
- 10 Cell adhesion and repulsion assay**
- This was carried out as previously described (Xiao et al, 1996). Briefly, 35mm tissue culture petri dishes (Becton Dickinson) were coated with methanol solubilized nitrocellulose (Lagenaur and Lemmon, 1987) and air-dried under a sterile hood. Proteins (2.5 µl of 12µM F3-Fc, CHL1-Fc, or different subcloned Notch1 extracellular fragments) were then applied to these dishes and incubated for 2 hours at 37°C in a humidified atmosphere. Subsequently, the dishes were washed three times with calcium- and magnesium-free Hank's balanced salt solution (CMF-HBSS) and blocked overnight with 2% heat-inactivated fatty acid-free BSA (Sigma) in CMF-HBSS (this blocking step is skipped in the repulsion test). The dishes were then rinsed again and the respective cells, such as OLN-93, murine Notch1 transfected HeLa cells (Logeat et. al., 1998), F3-transfected CHO cells, and NB-3-transfected CHO cells, were plated in 2 ml of chemically defined medium at a density of  $1.5 \times 10^6$  cells/ml. After 0.5 hour (in the adhesion test) or 12 hours (in the repulsion test), the dishes were gently washed three times with CMF-HBSS and the cells were fixed with 2.5% glutaldehyde in CMF-HBSS. Blockage of adhesion was carried out using anti-F3 (Gennarini et al, 1991; 1:100), polyclonal anti-Notch1 (Mitsiadis et al, 1995; 1:200) and anti-Notch2 (Mitsiadis et al, 1995; 1:200) antibodies. Cells adhering to the various spots were photographed and counted. The results were analyzed by Student's t test with  $p < 0.05$  and  $p < 0.01$  being

considered significant or highly significant difference, respectively.

#### Western Blot Analysis

- 5 Co-cultures of F3, TAX, TAG-1 and mock transfected CHO cells with OLN-93 cells were harvested and lysed by sonication in PBS containing the protease inhibitor cocktail tablet. After centrifuging at 100,000g for 1 hour at 4°C, the pellets were further solubilized in 2% Triton X-100. Subsequently, the
- 10 membrane portion of the cells, about 20µg protein per cell line, was analyzed by SDS-PAGE (8% gels; Laemmli, 1970) and Western blotting (Towbin et al, 1979) with antibodies against Notch1, Notch2, Notch3, myelin-associated glycoprotein, MAG (Yang et al, 1999) and proteolipid protein (PLP) (Jung et al, 1996).

15

#### Western blot analysis of developmental expression patterns

- The brain stem was dissected from embryos at E17 or neonates at between P0 and P21 of Wistar rats. The specimens were homogenized in 9 volumes of reducing sample buffer and boiled for 5 min. Each
- 20 10-µl aliquot of the homogenates was subjected to Western blot. Detection was carried out with ECL Western Blotting System (Amersham Biosciences).

#### Fusion Proteins

- 25 *Production of recombinant F3-Fc, Jagged1-Fc and CHL1-Fc fusion proteins.* Recombinant cDNA encoding mouse F3 with the GPI-anchor substituted with human IgG Fc was inserted at the Hind III-Not I sites of pDX with an amplification-promoting sequence (APS) (Hemann et al, 1994) and introduced into Ltk<sup>-/-</sup> cells. Fc fusion
- 30 proteins were purified as described (Shimizu et al, 1999).

- Production of recombinant NB-3-His fusion protein.* A soluble form of NB-3 recombinant protein, NB-3- His, was produced in HEK293 cells. The region of rat NB-3 cDNA encoding the signal sequence
- 35 of the GPI-anchor was substituted with 6x His followed by a

termination codon. The NB-3 cDNA thus manipulated was inserted into pREP4 between Hind III and BamHI sites, and the resulting vector was transfected into HEK293 cells. When the cells expressing NB-3-His reached confluence they were cultured in serum-free medium for a week. The culture medium was collected, concentrated and dialyzed against 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0. Ni-NTA resin (Qiagen) was added to the dialysate and incubated for at least 30 min. NB-3 recombinant protein was eluted from the Ni-NTA resin using 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole.

*Production of Notch1 GST fusion proteins.* Production of Notch1 GST fusion proteins has been described (Hu et al, 2003). Recombinant proteins comprising different regions of the extracellular domain of mouse Notch1 (mN1) were produced as follows. Primers with added Hind III and BspEI sites (underlined below) were used to amplify regions of mN1 cDNA (gift from Dr. Jeffrey Nye) in polymerase chain reactions.

20 N1.1 forward :5'-GGTGGAATTCTAATGCCACGGCTCCTG-3'  
reverse :5'-TTGAAGTTCCTCATCCGTGTTGATTT-3'  
N1.2 forward :5'-TGTGGAATTCTATGTGATCTGGGTGCC-3'  
reverse :5'-CGTCAAGTTCGTCATCGATGTCACTCT-3'  
N1.3 forward :5'-CTTGGAATTCTATGTGCTACCAGCCCC-3'  
25 reverse :5'-TTGAAGCTTGCCATTGATGACTGACT-3'  
N1.4 forward :5'-ACTGGAATTCTATGCCATCCCCCCTT-3'  
reverse :5'-AAGGAAGCTTCTGCGAGGGCAGCGGAG-3'

The regions amplified were N1.1 (nucleotides 79-1557; 1478 bp fragment encoding amino acids 27-519; EGF repeats 1-13), N1.2 (nucleotides 1324-2808; 1484 bp fragment encoding amino acids 442-936; EGF repeats 11-24), N1.3 (nucleotides 2575-4008; 1433 bp fragment encoding amino acids 859-1336; EGF repeats 22-34), and N1.4 (nucleotides 3751-5247; 1496 bp fragment encoding amino acids 1251-1749; EGF repeats 32-36, LNR repeats). Recombinant GST

fusion proteins were produced using pGEX-KG vector and purified as described (Xiao et al, 1996).

5 The PCR was performed on plasmid DNA in the presence of 1mM MgCl<sub>2</sub>. The cycles were: first five cycles at 93°C for 1 min, 45°C for 30 s and 72 °C for 1 min; the subsequent 30 cycles were carried out at 93 °C for 1 min, 50 °C for 30s and 72 °C for 1 min; and a final extension at 72 °C for 5 min. These amplified  
10 fragments were respectively used to create vectors pN1.1, pN1.2, pN1.3, and pN1.4 by restriction at at their extended HindIII and BspEI sites and ligation into similarly digested pGEX-KG vector. Transformed *E. coli* JM 109 cells were induced with IPTG, and the expressed GST-N1.1, -N1.2, -N1.3, and -N1.4 fusion proteins were purified using glutathione-agarose beads.

15  
*Production of recombinant NB-3-Fc proteins.* The signal sequence of the GPI-anchor was substituted with human IgG Fc followed by a termination codon. The cDNA thus manipulated was inserted into pREP4 between Hind III and BamHI sites, and transfected into 293T  
20 cells. NB-3-Fc was purified from the conditioned medium using Protein A-Agarose beads (Roche).

#### Immunohistochemistry

25 Following deep anaesthesia, adult Wistar rats were transcardially perfused with Ringer's saline followed by 4% paraformaldehyde in 0,1M phosphate buffer (PB; pH 7.4). Brain stem segments were harvested and post-fixed for 2 hours before being transferred into 0.1M PB containing 15% sucrose overnight. The tissue was frozen in O.C.T. compound (Tissue-Tek) and sagittal cryosections  
30 (10µm) were collected on gelatin-coated slides. For immunostaining, the slides were dried at 37 °C for 30 min, then immersed in cold acetone (-20 °C) for 15 minutes for permeabilization. Blockade of non-specific binding sites was carried out using 10% goat serum solution in 0.1M PB for 30

minutes at room temperature. Between steps involving antibodies, preparations were washed three times for 5 minutes each with 0.3% Triton X-100 in 0.1M PBS. All antibodies were diluted in 0.1M PB. Sections were double labeled. Tissue sections were typically  
5 first incubated overnight with the polyclonal primary antibody, followed by a secondary goat anti-rabbit antibody. Sections were then exposed to the monoclonal primary antibody and labeled with an anti-mouse secondary antibody. For examination, coverslips were applied and the slides viewed on a Zeiss laser scanning  
10 confocal microscope.

#### **Co-immunoprecipitation and GST Pull-Down Assay**

Rat brain membrane samples were prepared as described (Xiao et al, 1996) and incubated overnight at 4°C with antibody-coupled  
15 Protein A agarose beads (Roche) or glutathione-agarose beads (Sigma) bound to GST-N1.1, N1.2, N1.3 or N1.4. Captured proteins were eluted from beads with SDS-PAGE sample buffer, subjected to Western blot and detection using ECL reagent (Amersham).

20 NSCs and Rat brain membrane portions were prepared as described (Xiao et al, 1996) and incubated overnight at 4°C with NB-3-Fc and antibody-coupled Protein A-Agarose beads. Captured proteins were eluted from beads with SDS-PAGE sample buffer, subjected to Western blot and detection using ECL reagent.

25 *Immunoprecipitation of Caspr.* Chick or mouse brain lysate (800 µg), prepared as described (Zeng et al, 1999), was incubated with rabbit pre-immune serum (PI) or anti-Caspr serum followed by Protein A+G-Sepharose (Sigma). The immunoprecipitates were  
30 washed, resolved by SDS-PAGE and probed with anti-Caspr serum. For immunoblotting, pre-immune and immune sera were used at a 1:1000 dilution.

*Co-immunoprecipitation.* Brain membranes were prepared as

described previously (Isom et al, 1995). Membranes were solubilized in 2% Triton X-100 and the soluble fraction was incubated overnight at 4 °C with Notch antibodies (anti-Notch1 and anti-Notch2). Protein A Sepharose beads (50 µl of a 1:1 suspension) were then added and the incubation continued for a further 2 hours or overnight at 4 °C. The beads were washed with 50mM Tris HCL, pH 7.5, containing 0.1% Triton X-100 and protease inhibitors. Immunoprecipitated proteins were eluted from the beads with SDS-PAGE sample buffer and separated on 7.5% acrylamide SDS-PAGE gels. Proteins were transferred to nitrocellulose and probed with antibodies against F3 and NB-3. Chemiluminescent detection of immunoreactive bands was accomplished with ECL reagent. The reciprocal experiment was carried out using anti-Notch1 and anti-Notch2 antibodies to probe immunoprecipitates obtained using anti-F3 and anti-NB-3.

*GST Pull Down Assay.* Purified GST fusion proteins (mNotch 1.1, 1.2, 1.3 and 1.4) were coupled to Sepharose 4B beads (Amersham Pharmacia Biotech Inc) according to the manufacturer's instructions. Fresh cerebral hemispheres of adult rats were harvested and were solubilized in 2% Triton X-100. The homogenate was centrifuged at 13,000g for 60 minutes. The cleared lysate was then incubated for 45 min at room temperature with GST fusion protein bound to the beads. The beads were washed 3 times with lysis buffer and proteins were eluted with SDS-PAGE gel sample buffer, resolved on 7.5% SDS-PAGE, transferred to nitrocellulose and analyzed by Western blotting using anti-F3 and anti-NB-3 antibodies.

### 30 Culture of Primary OLS and OPCs

The inventor employed a glial cell separation technique in which oligodendrocytes are separated by Percoll gradient centrifugation as previously described (Colello and Sato-Bigbee, 1998). Briefly, an hour before plating cells, culture dishes (Falcon) were coated

with poly-L-lysine. Postnatal day 1-2 rats were sacrificed by decapitation and their cerebral hemispheres rapidly dissected out. Meninges and blood vessels were carefully teased away using microdissecting forceps. Hemispheres were transferred to ice-cold  
5 HEPES/HBSS in a petri dish and finely minced using a scalpel blade. The tissue was transferred to a 50ml conical tube and centrifuged for 5 minutes at 200xg after which the supernatant was discarded and the cell pellet resuspended in 10µg/ml DNAase I in HEPES/HBSS before subjecting it to centrifugation again. A  
10 single cell suspension was prepared by forcing the tissue through a 70 µm nylon mesh Falcon cell strainer, applied to a Percoll gradient, and the oligodendrocyte-containing fraction recovered after centrifugation. The cell suspension was diluted with HEPES/HBSS and oligodendrocytes were separated by differential  
15 adhesion. Oligodendrocytes obtained in this manner were suspended in chemically defined DMEM/ F12 medium.

*Culture of primary neurons, Ols, and astrocytes.* Neurons, OLs, and astrocytes of E17 Wistar rats were isolated and cultured as  
20 described (Itoh, 2002). OLs of P5-7 Wistar rat cerebella were obtained by Percoll gradient centrifugation (Colello and Sato-Bigbee, 1998) and OPCs were purified from P5-7 Wistar rat optic nerve (Bogler, 1997).

25 *Culture of NSCs.* Murine striatal neural stem cells were isolated from 14-d-old BALB/c mouse embryos (IFFA Credo, L'Arbresle, France) and cultured in DMEM/F12 with N2 supplement and EGF (20ng/ml) (Invitrogen) (Arsenijevic et al, 2001). To induce  
30 differentiation, the spheres were mechanically dissociated into single cells and treated with NB-3 or Jagged1 in culture medium without growth factor for 24 hrs, and then added into culture medium with 1% fetal calf medium.

#### Real-Time RT-PCR Analysis

Primary oligodendrocytes were plated onto protein spots of F3-Fc, NB-3-His and BSA in a similar fashion to the cell adhesion assay previously described. After 2 hours in culture, total RNA was extracted according to the manufacturer's instructions using a

5 QIAGEN RNeasy kit. Total RNA (0.5 µg) was reverse transcribed using the TaqMan RT Kit (Applied Biosystems). To measure the level of mRNA expression, we performed real-time quantitative PCR using the TaqMan system on an ABI PRISM 7700 Sequence Detection

10 Software (ABI). Primer and probe sequences are as follows.

Myelin Associated Glycoprotein (MAG)

Forward Primer : 5'-ATCCTGGCCACGGTCATC-3'

Reverse Primer : 5'-CACACCAGTACTCCCCATCGT-3'

15 Taqman Probe : 5'-CAGCTGGAACCTCCCTGCAGTGACG-3'

Proteolipid Protein (PLP)

Forward Primer : 5'-AGGCCAACATCAAGCTCATTCT-3'

Reverse Primer : 5'-CGGGATGTCCTAGCCATTTTC-3'

20 Taqman Probe : 5'-CCAAACAATGACACACCCGCTCCA-3'

Independent amplifications of each target and the GAPDH RNA were performed according to the manufacturer's instructions. Relative expression levels of each transcript were determined by employing

25 the comparative C<sub>T</sub> method as outlined in the ABI User's manual.

Total RNA from OLs or OLN cells was extracted using the QIAGEN RNeasy kit and treated with RNase-free DNaseI (Invitrogen) to eliminate genomic DNA. Samples were used for reverse

30 transcription with random hexamer primers using SuperScript First-Strand Synthesis System (Invitrogen) (Notch homologs, *Hes1*) or TaqMan RT Kit (Applied Biosystems) (MAG). β-actin and GAPDH were used as internal controls. Real-time PCR was performed using the SYBR Green PCR Master Mix (Notch homologs, *Hes1*) or TaqMan



system (MAG) on an ABI PRISM 7700 Sequence Detection System. The primers and TaqMan probes were designed using Primer Express Software (ABI) and sequences are available upon request. The raw data from at least four independent experiments were used to  
5 determine the relative expression levels of each transcript by employing the comparative  $C_T$  method (ABI User's manual).

#### Lipid Raft Assay

This was performed as described (Krämer *et al*, 1999).

10

#### Hes1 Luciferase Reporter Assay

OLN cells ( $1.5 \times 10^5$ /well) in 12-well dishes were used for Hes1 luciferase reporter assays. Cells were transiently transfected using Lipofectamine and Lipofectamine Plus reagents (Invitrogen).  
15 Each well received 0.2  $\mu$ g pGVB/Hes1 luciferase reporter plasmid together with various expression plasmids (0.1 or 0.2  $\mu$ g caN1; 0.3 or 0.6  $\mu$ g RBP-J, dn-RBP-J-myc, DTX1, DTX1-D1-HA, DTX1-D2-Flag). The  $\beta$ -galactosidase expression plasmid pCMV/ $\beta$ -Gal was included as internal control to monitor the transfection  
20 efficiency. Cells were lysed 24 hours post-transfection and assayed using the Steady-Glo Luciferase Assay Kit (Promega). The raw data from at least four independent experiments were used to determine the relative reporter activity.

25 The NSC experiments was performed as described (Hu *et al*, 2003). In brief, NSCs in 24-well dishes were transiently transfected with indicated constructs using Lipofectamine 2000 (Invitrogen). pCMV/ $\beta$ -Gal expressing  $\beta$ -galactosidase was cotransfected to monitor the transfection efficiency. Cells were subjected to  
30 luciferase assay 24 hours post-transfection using the Steady-Glo Luciferase Assay Kit (Promega). The raw data from at least four independent experiments were used to determine the relative reporter activity.

**Flow Cytometric Analysis**

The cells were trypsinized, washed with PBS and treated with FACSPerm (Becton Dickinson). Cells were stained with antibody to MAP2 (2a+2b), GFAP, CNPase and FITC-conjugated anti-mouse IgG1  
 5 and anti rabbit IgG, then analyzed by flow cytometer (FACScalibur, Becton Dickinson) with CELLQuest software Version (Becton Dickinson).

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